MODULATION OF DENDRITIC CELLS BY CHEMICAL-TREATED KERATINOCYTES: A ROLE FOR INTERLEUKIN 1 ALPHA

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DECLARATION

I, Irma Theres Matjeka, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Toxicity of keratinocytes, the major cell type in the epidermis, is found in several skin inflammatory diseases. Environmental influences such as chemicals, UV light, mechanical insult, or extreme temperatures can cause necrosis of keratinocytes leading to failure of local homeostasis, breaching of the skin barrier, and the potential for infection. In such situations dermal dendritic cells (DCs) may be the important sentinels by responding to danger signals released by the dying cells and potentially activating the adaptive immune system.

The aim of this project is the development of a human two cell model to study the influence of skin sensitisers and irritants on DCs in the presence of keratinocytes and test the hypothesis that DCs can respond directly to keratinocyte damage via the release of Interleukin-1 alpha (IL-1α) from the dying cells.

The coculture of sensitiser/irritant treated HaCaT cells with human monocyte-derived DCs led to activation (upregulation of cell surface markers and cytokine/chemokine production), and an increase in T cell stimulatory activity for the sensitisers and the irritants. The supernatant produced by sensitiser and irritant-labelled HaCaT cells was sufficient to induce DC maturation, again showing no difference between sensitisers and irritants. Both HaCaT cells and primary keratinocytes (EpiDerm^{TM}) released IL-1α after chemical exposure. IL-1α neutralising antibodies attenuated DC maturation induced by chemical-treated HaCaT supernatants. Furthermore the effect of IL-1α on DCs was studied.

Our *in vitro* model studying the interaction between keratinocytes and DCs suggests that both sensitisers and irritants can lead to cell damage in the skin which is associated with the release of IL-1α and possibly other danger signals. These can activate DCs in the underlying dermis and lead to activation of the adaptive immune system causing the clearance of infections or resulting in allergic reactions.

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ABBREVIATIONS

%	Percentage
ACD	Allergic contact dermatitis
AD	Atopic dermatitis
ADAM	A disintegrin and metalloproteinase
ADAMTS14	ADAM metallopeptidase with thrombospondin type 1 motif, 14
AHSG	Alpha-2-HS-glycoprotein
AIM2	Absent in melanoma 2
ALB	Albumin
AMP	Antimicrobial peptide
ANXA2	Annexin A2
AP-1	Activator protein-1
APC	Antigen-presenting cell
APOA2	Apolipoprotein A-II
APOBEC3	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3
AQP9	Aquaporin 9
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine-5'-triphosphate
ATP1B2	ATPase, Na+/K+ transporting, beta 2 polypeptide
B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1
BA	Benzoic acid
BDKRB2	Bradykinin receptor B2
bHLH	Basic helix-loop-helix
BIR	Baculociral IAP repeat
BSA	Bovine serum albumin
C1QB	Complement component 1, q subcomponent, B chain
C3	Complement component 3
C6orf124	Chromosome 6 open reading frame 124
CARD	Caspase recruitment domain
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cDC	Conventional DC
CDP	Common DC precursor
CFH	Complement factor H
CFP	Complement factor properdin
CH25H	Cholesterol 25-hydroxylase
CHS	Contact hypersensitivity
CHST2	Carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2
Ci	Curie
CLA	Cutaneous lymphocyte-associated antigen
CLEC9A	C-type lectin domain family 9A
CLP	Common lymphoid progenitor
CLR	C-type lectin receptors
CLU	Clusterin

CMIP	c-Maf-inducing protein
CMP	Common myeloid progenitor
COX-2	Type-2 cyclooxynase
cpm	Counts per minute
CRD	Carbohydrate recognition domains
CREB1	cAMP response element-binding 1
cRNA	Complementary RNA
CST7	Cystatin F (leukocystatin)
CTACK	Cutaneous T-cell-attracting chemokine
CTP	Cytidine triphosphate
ctrl	Control
CXCL	Chemokine (C-X-C motif) ligand
Cy	Cyanine
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Damage-associated molecular pattern
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic cell
DCNB	1,2-Dichloro-4-nitrobenzene
DC-SIGN	DC-specific intracellular adhesion molecule-3-grabbing non-integrin
dDC	Dermal dendritic cell
DiI	3’ tetra-methyl-indocarbocyanine perchlorate
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNBS	2,4-Dinitrobenzene-1-sulfonic acid
DNCB	1-Chloro-2,4-dinitrobenzene
DNFB	1-Fluoro-2,4-dinitrobenzene
DNP	Dinitrophenol
ds	Double stranded
DSP	Desmoplakin
DTH	Delayed type hypersensitivity
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
ELF5	E74-like factor 5
ELISA	Enzyme-linked Immunosorbent Assay
ELK1	E twenty-six (ETS)-like transcription factor 1
EPB41L1	Erythrocyte membrane protein band 4.1-like 1
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
F2	Coagulation factor II (thrombin)
$F(ab')_2$	Fragment antigen binding linked by disulfide bond
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FKSG2	Apoptosis inhibitor

FN1	Fibronectin 1
FOXF2	Forkhead box F2
FoxP3	Forkhead box P3
FSC	Forward scatter
g	Gram
G0S2	G0/G1switch 2
GABPA	GA-binding protein alpha chain
GAGE7	G antigen 7
GAL	Galanin prepropeptide
GalNAc	N-acetylgalactosamine
GBP5	Guanylate binding protein 5
G-CSF	Granulocyte colony-stimulating factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene ontology
GPR68	G protein-coupled receptor 68
GRO-α	Growth-regulated oncogene-alpha
GVHD	Graft versus host disease
H_2O	Water
Hand1-Tcfe2a	Heart- and neural crest derivatives-expressed protein 1-TF E2A
HBA	Hemoglobin, alpha 1
HBG	Hemoglobin, gamma A
HBSS	Hank's buffered saline solution
HBZ	Hemoglobin, zeta
HDAC4	Histone deacetylase 4
HIST2H2AA4	Histone cluster 2, H2aa4
HLA	Human leukocyte antigen
HMGB1	High-mobility group protein B1
HNF4A	Hepatocyte nuclear factor 4
HSP	Heat-shock protein
HSV	Herpes simplex virus
Hz	Hertz
ICAM-1	Intercellular adhesion molecule-1
ICD	Irritant contact dermatitis
icIL-1RA	Intracellular form of Interleukin-1 receptor antagonist
iDC	Immature dendritic cell
IDO1	Indoleamine 2,3-dioxygenase 1
iE-DAP	D-γ-glutamyl-*meso*-diaminopimelic acid
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
IFN	Interferon
IFNAR	Interferon-α/β receptor
Ig	Immunoglobulin
IGF2	Insulin-like growth factor 2 (somatomedin A)
IL	Interleukin

IL-18R	Interleukin 18 receptor
IL1A	Interleukin 1, alpha
IL1B	Interleukin 1, beta
IL-1NTP	IL-1α N-terminal peptide
IL-1RA	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-1RI	Type I IL-1 receptor
IL-1RII	Type II IL-1 receptor
IL1RN	Interleukin-1 receptor antagonist
iLC	Immature Langerhans cell
INHBA	Inhibin, beta A
iNOS	Inducible nitric oxide synthase
IP-10	IFN-inducible protein-10
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
ISG20	Interferon stimulated exonuclease gene 20kDa
ISRE	Interferon-stimulated regulatory elements
I-TAC	IFN-inducible T cell α-chemoattractant
JNK	c-Jun N-terminal kinase
K	Keratin
KC	Keratinocyte
KLF4	Kruppel-like factor 4 (gut)
KLRG-1	Killer cell lectin–like receptor G1
KRT	Keratin
l	Litre
LC	Langerhans cell
LFA	Leukocyte function associated antigen
LGALS2	Lectin, galactoside-binding, soluble, 2
LILRB5	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 5
LLNA	Local lymph node assay
LN	Lymph node
LOR	Loricrin
LOXL1	Lysyl oxidase-like 1
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LYPD3	LY6/PLAUR domain containing 3
M	Molarity
MAL	MyD88 adaptor-like
MAPK	Mitogen-activated protein kinase
MCAT	Malonyl CoA:ACP acyltransferase (mitochondrial)
MCP-1	Macrophage inflammatory protein
M-CSF	Macrophage colony-stimulating factor
MDA5	Melanoma differentiation associated protein 5
MDC	Macrophage-derived chemokine

mDC	Myeloid DC
MDP	Macrophage and DC precursor
MDS	Macrophage-derived chemokine
MFI2	Antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5
MGL	Macrophage galactose-type C-type lectins
MHC	Major histocompatibility complex
Mig	Monokine-induced by IFN-γ
min	Minutes
MIP	Macrophage inflammatory protein
ml	Millilitre
MMP	Matrix metalloproteinase
MMR	Macrophage mannose receptor
MPEG1	Macrophage expressed 1
MT	Metallothionein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response gene 88
MZF	Myeloid zinc finger
N	Normality
NAD	NACHT associated domain
NALP3	NACHT, LRR and PYD domains-containing protein 3
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHK	Normal human keratinocytes
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NLR	NOD-like receptors
NLRP3	NBD-, LRR-, PYD-containing protein 3
NOD	Nucleotide-oligomerisation domain
NUPR1	Nuclear protein 1
OASL	2'-5'-Oligoadenylate synthetase-like
OT-I cells	MHC class I-restricted, ovalbumin-specific, $CD8^+$ T cells
OVA	Ovalbumin
P2RX7	Purinergic receptor P2X, ligand-gated ion channel, 7
PAGE	Polyacrylamide-gel ectrophoresis
PAM	Peptidylglycine alpha-amidating monooxygenase
Pam_3CSK4	palmitoylated N-acyl-S-diacylglyceryl cysteine-serine-lysine-4
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Principal components
PCA	Principal component analysis
PD-1	Programmed death-1
pDC	Plasmacytoid DC
PDFG	Platelet-Derived Growth Factor

PD-L1	Programmed death-ligand 1
PE	Phycoerythrin
PFKFB3	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PHLDA1	Pleckstrin homology-like domain, family A, member 1
PI	Propidium iodide
PLAT	Plasminogen activator, tissue
Poly I:C	Polyinosine-polycytidylic acid
pre-cDC	Precursors of conventional DC
pre-DC	Precursor DC
PRR	Pattern-recognition receptors
PYD	Pyrin homology domain
PZP	Pregnancy-zone protein
QSAR	Quantitative Structural Activity Relationship
RAGE	Receptor for advanced glycation end products
RANTES	Regulated on activation, normal T cell expressed and secreted
RASD1	RAS, dexamethasone-induced 1
rh	Recombinant human
RIG-I	Retinoic acid-inducible gene-I
RIN	RNA integrity number
RLH	RIG-I-like helicases
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RPS4Y1	Ribosomal protein S4, Y-linked 1
RREB1	Ras Responsive Element Binding Protein 1
RT	Room temperature
S100	S100 calcium binding protein
S100A8	S100 calcium binding protein A8
SALT	Skin associated lymphoid tissue
SAR	Structural Activity Relationship
SARM	Sterile α- and armadillo-motif-containing protein
SDS	Sodium dodecyl sulfate
SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3
sIL-1RA	Secreted form of Interleukin-1 receptor antagonist
SILV	Silver homolog (mouse)
SLC6A19	Solute carrier family 6 (neutral amino acid transporter), member 19
SLS	Sodium lauryl sulfate
SOD3	Superoxide dismutase 3, extracellular
SP1	Specificity Protein 1
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)
SPOCD1	SPOC domain containing 1
SPP1	Secreted phosphoprotein 1
SRF	Serum response factor
ss	Single stranded

SSC	Side scatter
STAT1	Signal Transducers and Activators of Transcription 1
STEAP4	Six transmembrane epithelial antigen of prostate family member 4
TAL1-TCF3	T-cell acute lymphocytic leukemia protein 1 - TF 3
TBS	Tris-buffered saline
TCR	T cell receptor
TF	Transcription factor
TF	Transferrin
TFF1	Trefoil factor 1
TGF	Transforming growth factor
Th cell	T helper cell
TIP-DC	TNF- and iNOS-producing DC
TIR	Toll/IL-1 receptor
TIRAP	Toll-Interleukin-1 receptor domain-containing adaptor protein
TLR	Toll-like receptor
TM4SF19	Transmembrane 4 L six family member 19
TMB	Tetramethylbenzidine
TNBS	2,4,6-Trinitrobenzene-1-sulfonic acid
TNCB	1-Chloro-2,4,6-trinitrobenzene
TNF	Tumour necrosis factor
TNFRSF	Tumor necrosis factor receptor superfamily, member
TNP	Trinitrophenol
TPEN	N,N,N’,N’-tetrakis (2-pyridylmethyl)ethylenediamine
TRAF	TNF-receptor-associated factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cells
TRIF	TIR-domain-contatining adapter-inducing interferon-beta
TSLP	Thymic stromal lymphopoietin
U	Units
UBD	Ubiquitin D
UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)
USF1	Upstream transcription factor 1
UV	Ultraviolet
v	Volume
V	Volt
w	Weight
ZNF	Zinc finger
µl	Microlitre

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Chemical toxicity to keratinocytes triggers dendritic cell activation via an IL-1α path.

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1 INTRODUCTION

Allergic contact dermatitis (ACD) is, with a prevalence of 15-20% (Brasch et al., 2007; Nielsen et al., 2001), one of the most common skin diseases in the Western world. 80% of all occupational skin diseases are due to contact dermatitis (Cherry et al., 2000), which is often chronic, has a poor prognosis and great socioeconomic consequences for patients (Hutchings et al., 2001; Larsen et al., 2009). Contact allergy or contact hypersensitivity (CHS) is caused by an inflammatory immune reaction against a wide range of chemicals after prolonged or repeated contact with the skin. Contact allergy is clinically manifested as ACD which is developed at the site of contact with the chemical. Morphologically ACD is characterised by an eczematous reaction (e.g. induration-hardening of the tissue and erythema-redness) which occurs after 24-72 hours, classifying ACD as a delayed type hypersensitivity (DTH) or type IV hypersensitivity, which is mediated by antigen-specific T cells. Experimentally, ACD is measured in mouse models using very strong contact sensitisers that do not normally occur in the environment, such as 1-Chloro-2,4-dinitrobenzene (DNCB), 1-Fluoro-2,4-dinitrobenzene (DNFB), Oxazolone and 1-Chloro-2,4,6-Trinitrobenzene (TNCB). Their sensitising potential is assessed by skin painting and the reaction is referred to as CHS.

A CHS or ACD response is typically divided into two phases; the sensitisation phase and the effector phase (Averbeck et al., 2007) (Figure 1-1). The sensitisation phase is induced when the skin of a naive individual is exposed to a sufficient amount of contact allergen or skin sensitiser to stimulate a primary cutaneous immune response. Such chemicals are of low molecular weight (<500 Dalton) (Bos and Meinardi, 2000) and have the ability to easily penetrate the skin as they often bear lipophilic residues (Smith Pease et al., 2003). These chemicals are too small to induce an immunological response themselves. Therefore they need to be able to modify skin proteins (Aleksic et

al., 2007; Landsteiner and Jacobs, 1935), normally by covalent binding to it, in a way that the haptenated protein can form neoantigens that can lead to an immune response (Eisen et al., 1952). Electrophilic residues of the hapten can bind to nucleophilic residues of exposed amino acids, such as cysteine (which is the main amino acid involved in ACD), lysine, methionine, tyrosine or histidine (Aleksic et al., 2008; Basketter et al., 1995). Thiol groups and amino groups are the main targets (Becker et al., 2003; Parker et al., 1983). Free radical reactions can also lead to hapten-protein binding as well as direct non-covalent interactions with major histocompatibility complex (MHC) molecules, resulting in novel epitopes (Vocanson et al., 2009). Sensitising metal ions, such as nickel, cobalt or chromium can also induce ACD, but the reaction is somewhat different to organic chemicals. Metal ions do not form covalent bonds with proteins but generate noncovalent coordination protein-metal chelate complexes (Vocanson et al., 2009).

The sensitising chemical, or at least the part of it that binds to skin protein, is usually referred to as the hapten. Chemicals that are not reactive themselves, but have the intrinsic ability to become reactive via oxidation or metabolic processes, are called prehaptens or prohaptens respectively (Smith Pease et al., 2003). To induce sensitisation haptens need to possess inflammatory properties that lead to an activation of the innate immune system which delivers signals to antigen-presenting cells (APCs) that induce their maturation and migration. Furthermore, haptens need to be able to modify self-proteins in a way that leads to the formation and expression of neo-antigens that can elicit an immune response. Modified or haptenated proteins can be engulfed, processed and expressed on MHC class I and II by APCs, like Langerhans cells (LCs) or dermal dendritic cells (dDCs).

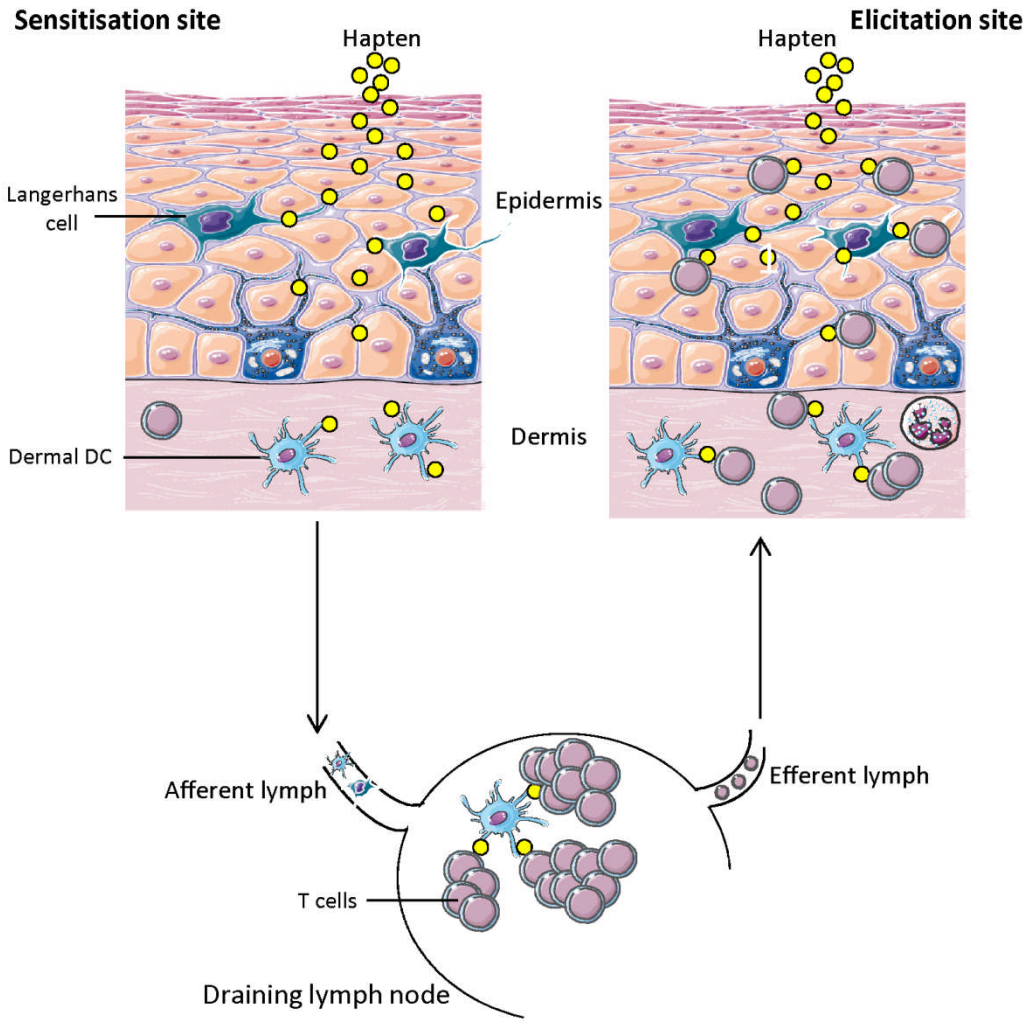


Figure 1-1: Mechanism of allergic contact dermatitis

Sensitisation phase: Haptens penetrate the skin and induce local danger signals. Langerhans cells in the epidermis and dermal dendritic cells (DCs) take up hapten-modified antigens, become activated and migrate to the draining lymph nodes via the afferent lymphatic vessels. In the lymph nodes the DCs present the haptenated peptides to circulating T cells. Specific T cell precursors bearing the cognate TCR become activated, expand clonally and develop into memory T cells. Effector memory T cells home back to the skin. **Elicitation phase:** Upon hapten encounter professional antigen-presenting cells as well as other skin cells can present haptenated antigens to memory T cells. Especially the activation of $CD8^+$ T cells initiates an inflammatory response through killing of hapten-presenting keratinocytes and the production of cytokines and chemokines, attracting another wave of immune cells. Part of the figure was used from "Medical Art Gallery" (Les Laboratoires Servier).

Activated APCs traffic to the lymph nodes where they then present the haptenated proteins to T cells. T cells bearing cognate T cell receptors (TCRs) in turn expand and form memory cells. This sensitisation (or afferent) phase is in most cases asymptomatic. The effector (or elicitation or efferent) phase is initiated when the skin of a sensitised

individual is subsequently exposed to the same hapten. The presentation of haptenated self-protein to memory T cells results in the activation and expansion of allergen-responsive T lymphocytes. The killing of keratinocytes (KCs) by $CD8^+$ T cells, as well as a mounted inflammatory response mediated by the release of cytokines, and other inflammatory mediators and the accumulation of mononuclear cells, leads to the clinical manifestation of ACD.

The allergenic potential of cosmetic ingredients has in the past been tested in the local lymph node assay (LLNA) using mice. With ACD being one of the most common skin diseases especially in the Western world, the development of an *in vitro* alternative to the LLNA is an urgent need. For the development of an *in vitro* assay for skin sensitisation it is important to understand the molecular and cellular processes leading to ACD.

1.1 The skin immune system

The skin is the largest organ of the human body and functions as a protective barrier against microbial pathogens and physical and chemical insult, as well as maintaining internal homeostasis by preventing hypothermia and dehydration. The concept of the skin-associated lymphoid tissue (SALT), where invading pathological agents are actively recognised by antigen presenting cells and cleared by T lymphocytes shuttling between skin draining lymph nodes and the skin was first postulated in 1983 by Streilein (Streilein, 1983) and later refined to the “skin immune system” (Bos et al., 1987). The importance of immune surveillance in maintaining skin homeostasis is evident by the increase in cutaneous malignancies and infections in immunosuppressed individuals (Lugo-Janer et al., 1991; Uthayakumar et al., 1997), as well as the many inflammatory disorders, including atopic dermatitis (Bonness and Bieber, 2007) and ACD (Freudenberg et al., 2009), psoriasis (Lowes et al., 2007), vitiligo (Glassman,

2010), alopecia areata (Gregoriou et al., 2010) and lichen planus (Roopashree et al., 2010) that result from an inappropriate immune response.

The human skin consists of the epidermis and the dermis (Figure 1-2). The **epidermis**, the outermost layer of the skin, mainly contains KCs which in their differentiated state produce intermediate filaments, the keratins, to provide strength. The innermost layer of the epidermis, the **stratum basale**, contains a single layer of undifferentiated KCs which constantly divide in order to replenish the epidermis with new cells. Newly divided cells move outwards into the **stratum spinosum** (also called the prickle cell layer) where numerous desmosoms connect the keratins of neighbouring cells. The KCs of the **stratum granulosum** are rich in lipids and keratins and form a waterproof barrier (Nestle et al., 2009). The outermost layer of the epidermis, the **stratum corneum**, contains dead KC-derived cells (corneocytes), which are devoid of any organelles but packed with keratins to ensure the barrier functions of the epidermis (Proksch et al., 2008). Melanocytes which produce the pigment melanin and LCs which are antigen-presenting innate immune cells are also found in the epidermis. $CD8^+$ T cells of the adaptive immune system can be found in the stratum basale and stratum spinosum (Nestle et al., 2009). The **dermis** which lies underneath the epidermis shows a more complex organisation. Adipocytes and fibroblasts are the major types of non-immune cells, with the latter playing a crucial role in producing components of the extracellular matrix (collagen, elastin) that give the dermis its´ strength and resilience. Many immune cells, including $CD4^+$ T cells, γδ Tcells, natural killer T (NKT) cells, dDCs, plasmacytoid DCs (pDCs), macrophages and mast cells can be found (Nestle et al., 2009). Several blood and lymphatic vessels traverse the dermis allowing entry and egress of these immune cells.

Cutaneous immune surveillance is underwritten by skin resident cells in acting as sentinels towards danger signals including mechanical, chemical or ultraviolet (UV) light induced damage as well as invading pathogens. KCs and LCs in the epidermis and DC, macrophages and mast cells in the dermis can release antimicrobial peptides (AMPs), cytokines and chemokines to initiate an immune response (Murphy et al., 2000; Yang et al., 2001).

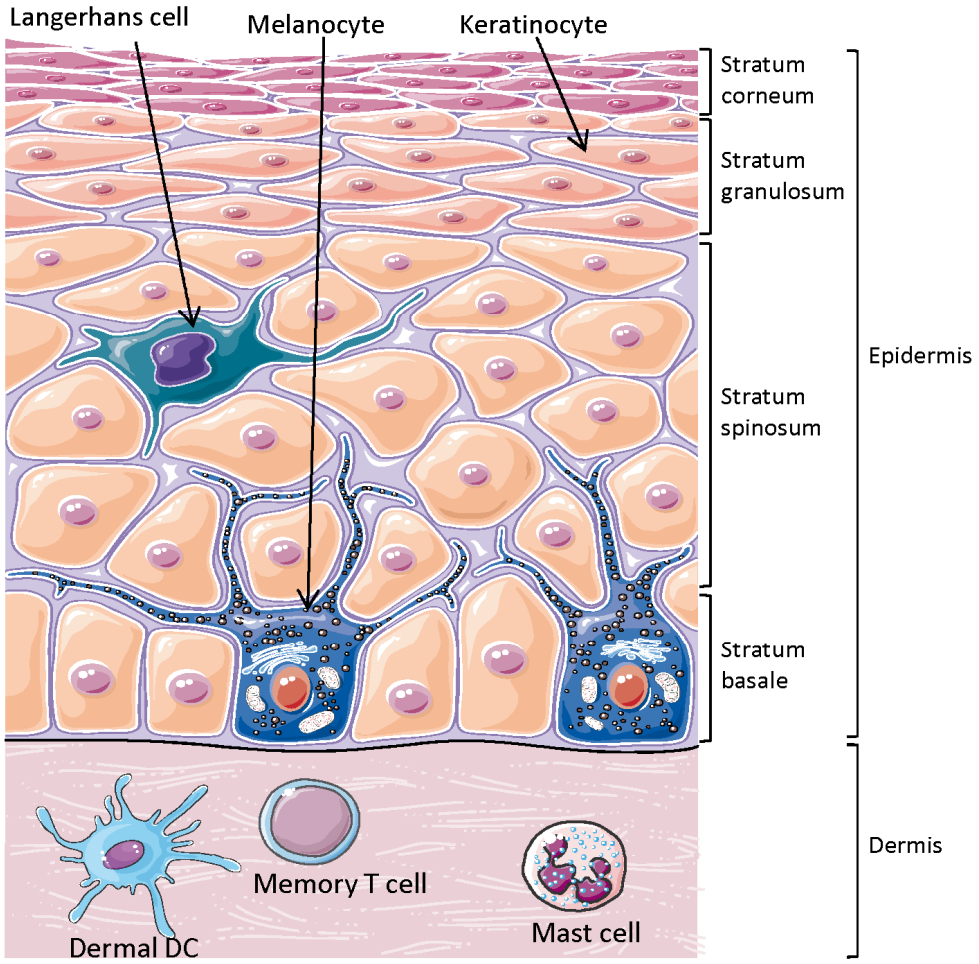


Figure 1-2: Anatomy of the skin.

The outer layer of the skin, the **epidermis** mainly consists of keratinocytes and can be subdivided according to their differentiation status. Immune cells, such as Langerhans cells and T cells can be found. The **dermis** shows a wider variety of immune cells. T cells, mast cells, macrophages and dermal dendritic cells (DCs) are resident. Adapted from "Medical Art Gallery" (Les Laboratoires Servier).

1.2 Keratinocytes

The importance of the main cell type in the epidermis, the KC, in contributing to cutaneous immune responses, is often underrated although they possess many immunomodulatory properties.

1.2.1 Keratinocyte activation

The recognition of invading pathogens, either directly via pathogen-associated molecular patterns (PAMPs) or indirectly through the release of danger signals/damage-associated molecular pattern (DAMPs) by damaged tissue, plays a crucial role in the control of infections as it leads to activation of the adaptive immune system and a clearance of infection. Tight regulation of these pathways is crucial as uncontrolled activation can lead to unrestrained inflammation, autoimmune disorders or even death.

KCs are equipped with germline encoded pattern-recognition receptors (PRR) which can recognise conserved **PAMPs** displayed by different microbes. A number of different families of PRR are known, such as toll-like receptors (TLRs), nucleotide-oligomerisation domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and retinoic acid-inducible gene-I (RIG-I)-like helicases (RLHs) (see Table 1-1).

Group	PRR	Ligand	Organism	References
TLR	TLR1/2	Triacylated lipoproteins	Bacteria, mycobacteria	(Takeuchi et al., 2002)
	TLR2	Lipoteichoic acid, peptidoglycan, lipoglycans	Gram(+) bacteria, measles virus	(Takeuchi et al., 1999)
	TLR2/6	Diacylated lipoproteins, zymosan	Mycoplasma, group B Staphylococcus, yeast	(Ozinsky et al., 2000; Takeuchi et al., 2001)
	TLR3	ds RNA	Viruses	(Alexopoulou et al., 2001)
	TLR4	lipopolysaccharide	Gram (-) bacteria	(Medzhitov et al., 1997; Park et al., 2009)
	TLR5	Flagellin	Flagellated bacteria	(Hayashi et al., 2001)
	TLR7	ss RNA	RNA viruses	(Diebold et al., 2004; Heil et al., 2004)
	TLR8	ss RNA	RNA viruses	(Heil et al., 2004)
	TLR9	Unmethylated CpG DNA	Bacteria and mycobacteria	(Hemmi et al., 2000)
	TLR10	unknown	Unknown	(Chuang and Ulevitch, 2001)
NLR	Nod1	Peptidoglycan (iE-DAP)	Gram(-) bacteria	(Chamaillard et al., 2003)
	Nod2	Peptidoglycan (MDP)	Bacteria	(Girardin et al., 2003)
	NALP1	MDP, anthrax toxin		(Martinon et al., 2009)
	NALP3	MDP, toxins, DNA, danger signals (uric acid crystals, ATP)	Bacteria	(Martinon et al., 2009)
	NAIP5	Flagellin	Gram(-) bacteria	(Mariathasan and Monack, 2007)
CLR	Dectin-1	β-Glucan	Fungi	(Brown and Gordon, 2001; Goodridge et al., 2011)
	DC-SIGN	Mannose, fucose	Viruses, mycobacteria	(Geijtenbeek et al., 2000b; Gringhuis et al., 2007)
	MMR	Mannose, fucose, N-acetylglucosamine	Viruses, bacteria, yeast	(Taylor and Drickamer, 1993)
RLH	RIG-I	ssRNA	Viruses	(Pichlmair et al., 2006)
	MDA-5	dsRNA	Viruses	(Kato et al., 2006)

Table 1-1: Characteristics of human PRRs

TLRs were the first PAMPs to be discovered and are therefore the best characterised. Ten different human TLRs and 11 mouse TLRs have been described, which show a broad specificity for PAMPs shared by a large group of pathogens, such as bacteria, fungi and viruses. Activation of TLRs leads to binding of adapter molecules

which facilitate a downstream signalling cascade resulting in the activation of transcription factors, a change in the transcriptional profile and the induction of an inflammatory response, mainly through the release of numerous chemokines (Iwasaki and Medzhitov, 2004; Jenner and Young, 2005). This will be further dissected in chapter 1.3. TLRs are expressed on many different immune cells, including DCs, macrophages, neutrophils, mast cells and B cells, as well as non-immune cells including fibroblasts and epithelial cells (Iwasaki and Medzhitov, 2004). KCs have been shown to express several TLRs, either on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) or in endosomes (TLR3 and TLR9) (Lebre et al., 2006). Furthermore it was shown that double stranded ribonucleic acid (dsRNA) can induce the endosomal expression of TLR7 (Kalali et al., 2008). Several TLRs have been implicated in CHS.

Another important set of innate immune receptors complementary to the TLRs are the **NLRs**, which act in the cytosol, recognising mainly bacterial products. Their genes are composed of three domains (1) at the N-terminus: pyrin homology domain (PYD), or caspase recruitment domain (CARD), or Baculociral IAP repeat (BIR); (2) at the intermediary region: nucleotide-oligomerisation domain (NOD), or NACHT, or NACHT associated domain (NAD); and (3) a C-terminal leucine-rich repeat (LRR) domain (Ishii et al., 2008). NOD1 and NOD2 sense muropeptides released from bacterial peptidoglycan leading to nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein kinase (MAPK) activation (Inohara et al., 2000; Kobayashi et al., 2005). NOD1 is activated by D-γ-glutamyl-*meso*-diaminopimelic acid (iE-DAP), mainly found in Gram-negative bacteria; whereas NOD2 detects muramyl dipeptides, which is common amongst Gram-positive and Gram-negative bacteria (Chamaillard et al., 2003; Martinon et al., 2009; McDonald et al., 2005). The other NLRs form large protein complexes with adaptor apoptosis-

associated speck-like protein containing a CARD (ASC) and caspaspe-1, which are referred to as inflammasomes. Activation of the inflammasomes by bacterial pore-forming toxins, muramyl dipeptides, deoxyribonucleic acid (DNA), uric acid crystals or extracellular Adenosine-5'-triphosphate (ATP) results in activation of caspase-1, to cleave pro-IL-1β and pro-IL-18 into their mature forms (IL-1β and IL-18 respectively), which can be released from the cells (Mariathasan and Monack, 2007; Martinon et al., 2009). Exposure of the skin to UV irradiation (Feldmeyer et al., 2007) or contact sensitisers (Watanabe et al., 2007) has been shown to activate the inflammasome in KCs and the release of IL-1β and IL-18. Furthermore, ASC and NACHT, LRR and PYD domains-containing protein 3 (NALP3)-deficient mice exhibited an attenuated response towards contact sensitisers (Watanabe et al., 2007). Cytosolic DNA can act as a danger signal in psoriasis by activating the absent in melanoma 2 (AIM2) inflammasome in KCs (Dombrowski et al., 2011; Fernandes-Alnemri et al., 2009). A second pathway of self-DNA recognition might also contribute to the pathogenesis of psoriasis. Aggregates of self-DNA and antimicrobial peptides have been shown to induce type I IFN production by pDCs via stimulation of TLR9 (Lande et al., 2007).

CLRs are transmembrane PRRs binding distinct carbohydrates, such as mannose, fucose or glucan structures via highly conserved carbohydrate recognition domains (CRD). These carbohydrate structures are usually found on viruses, fungi, bacteria, including mycobacteria, and helminths. CLRs, such as Dectin-1, Dectin-2, mannose receptor, DC-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) or Langerin are mainly expressed on DCs and monocytic cells (Figdor et al., 2002). Further to antigen uptake, CLRs have been attributed a role in antigen cross presentation, cell trafficking and T cell activation (Burgdorf et al., 2006; Geijtenbeek et al., 2000a; Geijtenbeek et al., 2000c). In mice it was found that dDCs expressing

macrophage galactose-type C-type lectins 2 (MGL2), a CLR that binds N-acetylgalactosamine (GalNAc)-polymers, are sufficient to induce CHS after adoptive transfer (Kumamoto et al., 2009).

The **RLHs** melanoma differentiation associated protein 5 (MDA5) and RIG-I are cytosolic proteins found in most cells, except pDCs, recognising RNA viruses and inducing an antiviral immune response by inducing the production of type I interferons (IFNs) (Kato et al., 2006; Pichlmair et al., 2006). Furthermore, viral/bacterial DNA can be detected by another intracellular receptor called DNA-dependent activator of IFN-regulatory factors (DAI) (Takaoka et al., 2007). KCs have been shown to express RIG-I and MDA-5 and are thought to play a role in anti-viral defence (Kalali et al., 2008).

TLRs and NLRs not only play a role in recognising PAMPs, but they have also been implicated in responding to danger signals. These are also referred to as damage-associated molecular patterns (**DAMPs**). The "danger" model was proposed by Matzinger (Matzinger, 1994) and Ibrahim (Ibrahim et al., 1995). They postulated that the immune system had not evolved to recognise infection *per se*, but to respond to signals that are released following tissue damage. This might explain why non-infectious agents, such as transplanted tissue or tumours, can lead to strong immune responses, as well as the occurrence of autoimmune disorders. DAMPs are endogenous host molecules that are normally sequestered intracellularly, but released into the extracellular milieu through necrotic cell death. Like PAMPs they lead to inflammation as well as activation of the adaptive immune system in order to ensure the complete clearance of tissue destruction and the prevention of excessive inflammation. That pathogens and trauma evoke similar responses may not be that surprising as pathogens can easily breach wounds, as well as induce cell damage themselves (Bianchi, 2007).

Heat-shock proteins (HSPs) were the first host proteins that were attributed adjuvant properties. Purified HSPs were shown to augment anti-tumour responses in mice (Feng et al., 2003; Udono and Srivastava, 1993) as well as activate DCs *ex vivo* (Basu et al., 2000; Binder et al., 2000). However, it is not clear whether these adjuvant properties are due to the HSPs themselves or if microbial contamination, such as lipopolysaccharide (LPS) produced this effect (Bausinger et al., 2002). It has been suggested that HSP60 binds LPS to induce synergistic effects in APCs (Osterloh et al., 2007). **Monosodium urate crystals** have been found to be a danger signal that can activate Caspase-1 via the NALP3 inflammasome, leading to the production of active IL-1β and IL-18 (Martinon et al., 2006). Moreover, inflammasome- and IL-1 receptor (IL-1R)-deficient mice showed an impaired neutrophil influx, suggesting a crucial role of the inflammasome in alerting the innate immune system to respond to danger signals (Martinon et al., 2006). This pathway seems to play an important role in inducing gouty inflammation.

High-mobility group protein B1 (HMGB1) was originally described as a non-histone binding nuclear protein which functions in somatic cells in transcriptional regulation, stabilisation of nucleosomes and DNA bending, as reviewed in (Stros, 2010). Later on it was shown to be a late mediator during sepsis (Wang et al., 1999b). HMGB1 can be actively released by activated monocytes, macrophages or DCs or it can passively leak out from dying cells (Bonaldi et al., 2003; Scaffidi et al., 2002). However, it is only released from necrotic cells, but retained in the nucleus during apoptosis (Scaffidi et al., 2002). It exerts its function by activating monocytes, macrophages and DCs to release proinflammatory mediators (Andersson et al., 2000; Dumitriu et al., 2007; Yanai et al., 2009; Yang et al., 2007). HMGB1 has been implicated to trigger a variety of receptors. It has been suggested that it signals via

TLR2 and TLR4 (Yang et al., 2010; Yu et al., 2006), although other people reported contradicting results (Tian et al., 2007). HMGB1 has also been suggested to act as a universal sentinel for nucleic-acid-mediated innate immune responses by facilitating optimal signal through TLR3, TLR7 and TLR9 (Tian et al., 2007; Yanai et al., 2009). Furthermore, HMGB1 has been shown to signal through receptor for advanced glycation end products (RAGE) (Dumitriu et al., 2007; Scaffidi et al., 2002; Tian et al., 2007; Yang et al., 2007), as well as danger signals of the S100 family (Hofmann et al., 1999). Several DAMPs have been discovered, although their individual importance or redundancy remains to be investigated. Other endogenous DAMPs include the just mentioned S100 proteins, DNA, RNA, ATP and AMPs. As mentioned before, most DAMPs are intracellular agents that get released from dying cells. However DAMPs can also be generated indirectly from extracellular molecules (mainly the extracellular matrix) as a result of proteolysis by enzymes released during necrosis. Furthermore dying cells can release intracellular stores of pro-inflammatory cytokines, such as IL-1α or IL-33. Although, they are not considered to be DAMPs, strictly speaking, they are able to elicit a sterile inflammatory response (see chapter 1.3). Furthermore C-type lectin domain family 9A (CLEC9A) has been identified as a receptor on DCs sensing necrotic cells, although its ligand has not been identified yet (Sancho et al., 2009). Chen et al., showed that it may be possible for the immune system to at least distinguish some DAMPs from PAMPs and specifically downregulate their pro-inflammatory activities. Cluster of differentiation (CD24) binding to HMGB1, HSP70 or HSP90 has been shown to attenuate NFκB activation and cytokine production by binding to Siglec G/10 (Chen et al., 2009).

As the major cell type in the epidermis and the barrier to the external world KCs play a crucial role in releasing danger signals. It has been shown that necrotic KCs

release HSP, HMGB1 and IL-1. As previously mentioned KCs can produce AMPs in response to pathogenic invasion and damaged epithelia. The main AMPs produced are β-defensins and cathelicidins which are involved in the direct killing of pathogens by inducing holes into the microbial lipid bilayer. Furthermore AMPs attract host immune cells and alter their gene expression profile, promote wound healing and induce cytokine and chemokine production (Lai and Gallo, 2009). Dysregulation of AMPs regulation has been associated with a number of skin diseases. In psoriasis exaggerated expression of AMPs is believed to participate in breaking tolerance to self-DNA and herein leading to autoimmunity whereas decreased expression in atopic dermatitis can countenance superinfection (Lai and Gallo, 2009; Lande et al., 2007). The role of AMPs in CHS is less clear. Increased expression of the cathelicidin LL-37 was found in KCs of skin samples of patients with nickel allergies (Frohm et al., 1997). Whereas in a mouse model using DNFB to induce CHS, deletion of the cathelicidin gene increased the contact allergy response and topical application of cathelicidin before sensitisation attenuated the allergic response (Di Nardo et al., 2007). The reduced CHS response was suggested to be due to a TLR4 dependent reduction of DC responsiveness (Di Nardo et al., 2007).

1.2.2 Keratinocytes as non-professional antigen-presenting cells

Activation of KCs by IFNγ (most likely derived from T cells, natural killer (NK) cells or NKT cells) leads to upregulation of MHC class I and II and the costimulatory molecules CD40 and CD54 (intercellular adhesion molecule-1 (ICAM-1)), raising the question if KCs can function as APCs (Black et al., 2007; Wittmann and Werfel, 2006). Several *in vitro* and *in vivo* models suggest that KCs generally induce T cell tolerance or anergy as opposed to T cell activation (Bal et al., 1990; Gaspari and Katz, 1991; Nickoloff et al., 1993; Niederwieser et al., 1988). B7-H1 (programmed death-ligand 1

(PD-L1)/CD274), which can be induced on KCs by several cytokines, may play a role in downregulating $CD8^+$ T cell effector functions by binding the coinhibitory receptor programmed death-1 (PD-1) on skin homing T cells (Ritprajak et al., 2010). T cell activation may also be inhibited by binding of E-cadherin, which is highly expressed by KCs, to killer cell lectin-like receptor G1 (KLRG1) expressed on senescent T cells (Henson et al., 2009). However, IFNγ-stimulated KCs can function as accessory cells for T cell proliferation against bacterial superantigens, whereby a leukocyte function associated antigen (LFA)-1/ICAM costimulatory signal is crucial (Nickoloff et al., 1993). Despite a lack of CD80/CD86 expression it was shown that human primary KCs can induce peptide-specific memory responses in $CD4^+$ and $CD8^+$ T cells (Black et al., 2007). Peptide was actively processed and Th1 as well as Th2 responses could be induced (Black et al., 2007). Therefore, it was thought that KCs are unable to stimulate naive T cell responses, but may present to antigen experienced T cells as part of a recall immune response. A recent study using a graft versus host disease (GVHD) model, however, showed that ovalbumin (OVA)-expressing KCs were able to activate naive MHC class I-restricted, ovalbumin-specific, $CD8^+$ T cells (OT-I cells) *in vivo*, independently of LCs and dDCs, as well as *in vitro*. Nevertheless, the KCs failed to initiate an allogeneic epidermal lymphocyte reaction (Kim et al., 2009), consistent with previous reports.

1.2.3 Cytokine and chemokine secretion by Keratinocytes

Inflammatory skin diseases, such as psoriasis, atopic dermatitis and ACD are characterised by infiltration of activated T cells into the dermis and epidermis, where they modulate the local environment by the release of cytokines. KCs can also produce numerous cytokines, including IL-1, IL-3, IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF),

macrophage colony-stimulating factor (M-CSF), transforming growth factor (TGF)-α, TGF-β, tumour necrosis factor (TNF)-α and platelet-derived growth factor (PDFG), upon activation with proinflammatory cytokines, such as IFN-γ and TNF-α (Ansel et al., 1990). Furthermore, they produce the C-X-C chemokines growth-regulated oncogene-alpha (GRO-α, CXCL1), IL-8 (CXCL8), monokine-induced by IFN-γ (Mig, CXCL9), IFN-inducible protein-10 (IP-10, CXCL10) and IFN-inducible T cell α-chemoattractant (I-TAC ,CXCL11); and the C-C chemokines I-309 (CCL1), macrophage inflammatory protein-1 (MCP-1, CCL2), regulated on activation, normal T expressed and secreted (RANTES, CCL5), macrophage inflammatory protein (MIP)-3α (CCL20), macrophage-derived chemokine (MDC, CCL22) and cutaneous T-cell-attracting chemokines (CTACK, CCL27) (Albanesi et al., 2001; Lebre et al., 2003) after stimulation, which regulate migration of diverse types of leukocyte in the skin lesion. *In vitro* studies suggest that primary KCs are more sensitive to activation by T helper (Th)1 cells than Th2 cells. Activated KCs in turn release chemokines which preferentially recruit Th1 cells, resulting in selective amplification of the Th1 response (Albanesi et al., 2001). Furthermore, KCs can be directly activated by allergens and irritants. Hexavalent chromium for example induces reactive oxygen species (ROS) formation, Akt, NFκB and MAPK activation as well as TNF-α and IL-1α production in KCs (HaCaT cells) (Wang et al., 2010). Activated KCs can also lead to DC maturation as well as modulate their Th polarising function (Lebre et al., 2003). Proinflammatory cytokines (IL-1α, TNF-α) and Th2 cytokines (IL-4, IL-13) synergise in the production of thymic stromal lymphopoietin (TSLP) from KCs, which can activate DCs (Bogiatzi et al., 2007). This is mainly found in atopic dermatitis. Furthermore, it was shown that IFN-γ is crucial during the sensitisation and the effector phase in CHS to induce production of Th1 cytokines from KCs (Mori et al., 2008). KCs contain large quantities of biologically active IL-1α, which can be released in response to a range of stimuli. Synthesis and

release of TNF-α by KCs is induced by phorbol esters, LPS, contact sensitisers and UV irradiation. IL-1 stimulates KCs to produce IL-6 and IL-8, which amplify a proinflammatory response (Newby et al., 2000). KCs have been shown to produce proinflammatory cytokines and chemokines in response to contact sensitisers (Enk and Katz, 1992; Goebeler et al., 2001). Sugita et al. show in a mouse model of CHS that KC-derived soluble factors (including IL-1α, TNF-α and GM-CSF) play a crucial role in the activation of LCs (Sugita et al., 2007). KCs produce IL-23 in response to nickel (Larsen et al., 2009). IL-23 induces IFN-γ expression in memory T cells and is therefore important for the maintenance of a type I immune response. In healthy skin IL-23 is expressed by KCs, DCs, LCs and macrophages (Piskin et al., 2006). Furthermore, KCs can release matrix metalloproteinases (MMPs), which are proteolytic enzymes involved in tissue remodelling and extracellular matrix (ECM) turnover. Nickel induces expression of MMP2, a key enzyme for degrading type IV collagen, which is a major component of the basal membrane (Perfetto et al., 2007). MMP2 and MMP9 are overexpressed in involved skin of ACD patients after rechallenge (Giannelli et al., 2002).

1.2.4 Experimental model: HaCaT cells

As an experimental model for KCs we used the human cell line HaCaT. It is derived from histologically normal skin obtained from the distant periphery of a melanoma, whereby the KCs transformed spontaneously *in vitro* (Boukamp et al., 1988). Cell lines are of course always a compromise compared to primary cells and HaCaT cells for example have been shown to have mutations in the p53 gene (Lehman et al., 1993). However, the availability, convenience and cost and time effectiveness support the use of cell lines.

HaCaT cells are widely used as a model for KCs as they are very well studied and show a near normal phenotype (Boukamp et al., 1988; Deyrieux and Wilson, 2007; Lemaitre et al., 2004). They can be maintained in an undifferentiated (basal) state by culturing them at low density and in low calcium concentrations, whereby they express the basal keratins K5 and K14. The presence of high calcium concentrations and high confluency leads to differentiation, characterised by a very compact growth and the expression of K1, K10 and involucrin (Boukamp et al., 1988; Deyrieux and Wilson, 2007; Lemaitre et al., 2004). Furthermore, HaCaT cells grown in organotypic cultures or grafted onto nude mice showed normal keratinisation and stratification, forming an epidermis (Boukamp et al., 1988).

1.3 Interleukin-1

IL-1 is a proinflammatory cytokine with multiple effects on proliferation, differentiation and function of immune and non-immune cells (Dinarello, 1996). The IL-1 family consists of 11 members: IL-1α, IL-1β, IL-1RA, IL-18, IL-33 and IL-1F5 – IL-1F10. The two best studied isoforms are IL-1α and IL-1β. In context of this thesis, when talking about IL-1α and IL-1β, we will refer to them as IL-1, as they signal through the same receptor (IL-1RI) and are thought to have similar biological activities in solution. The sequence homology of IL-1α and IL-1β is very low (20-30%), however the 3-dimensional structure is very similar. In contrast to most cytokines IL-1 lacks a signal sequence and is therefore not secreted via the ER-Golgi pathway. Both forms are synthesised as precursors which need to be cleaved into their mature forms by proteases before they can be actively released.

1.3.1 Interleukin-1 beta

1.3.1.1 The function of IL-1 beta

IL-1β is mainly produced by blood monocytes, tissue macrophages and DCs. B lymphocytes and NK cells can also produce IL-1β; however, fibroblasts and epithelial cells generally do not. IL-1β plays pleitropic roles in infectious autoimmune diseases e.g. development of fever and production of acute-phase proteins (Martinon et al., 2002). Injection of IL-1 into humans resulted in fever, headache, nausea, myalgias and arthralgias (Dinarello, 1996). Furthermore, hypotension, increase in pro-inflammatory mediators, such as IL-6 and enhanced hematopoiesis, especially augmented neutrophil and monocyte counts were observed. The genes inducible nitric oxide synthase (iNOS), type-2 cyclooxygenase (COX-2) and type-2 phospholipase A_2 (PLA_2) seem to be particularly sensitive to IL-1. They encode for nitric oxide, prostaglandin, leukotrienes, and platelet-activating factor, promoting further inflammation (Dinarello, 1996). The adjuvant alum has been reported to stimulate IL-1β release through activation of the NALP3 inflammasome (Eisenbarth et al., 2008). IL-1β deficient mice do not show any spontaneous diseases. However, in different disease models, such as Zymosan peritonitis, turpentine-induced inflammation, systemic lupus erythematosus and collagen-induced arthritis, the disease severity and inflammation are generally attenuated, shown for example by reduced expression of IL-6 and chemokines as well as reduced neutrophil infiltration and fever (Dinarello, 2009). These findings confirm the crucial role of IL-1β in local and systemic inflammation. Autoinflammatory diseases have been defined as a group of inflammatory diseases, which are mainly mediated by IL-1β. Some of these diseases show mutations in proteins that comprise the inflammasome, but generally they are characterised by their periodicity. Autoinflammatory diseases are often triggered through environmental stress and, in

contrast to autoimmune diseases, do not respond to anti-TNF-α or anti-IL-12/IL-23 treatment or therapies using CTLA4-Ig. Instead they respond to IL-1 antagonists, such as anakinra, which has been successfully used to treat patients with inherited periodic fever syndromes, such as Muckle-Wells syndrome, familial cold autoinflammatory syndrome (both caused by mutations in NALP3) or gout (Hawkins et al., 2003; Hoffman et al., 2004; So et al., 2007). Anakinra has also been used to treat autoimmune diseases, such as rheumatoid arthritis, or type 2 diabetes.

IL-1β plays an important role in the differentiation and maintenance of Th17 cells, which may contribute to both autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis and chronic inflammatory diseases such as adult-onset Still's disease (Chen et al., 2010). Mice carrying a point mutation in NALP3 which has been associated with Muckle-Wells syndrome showed increased production of IL-1β by APCs as well as augmented Th17 responses (Meng et al., 2009).

Originally, IL-1 (IL-1α and IL-1β) was suggested to act directly on T lymphocytes and stimulate their proliferation in the presence of antigen. IL-1 was therefore termed lymphocyte activating factor. However, it has been shown subsequently in several independent studies that IL-1 does not stimulate T cells, but rather activates the accessory function of human and murine DCs to augment T cell proliferation (Bhardwaj et al., 1989; Eriksson et al., 2003; Heufler et al., 1988; Inaba et al., 1988; Koide et al., 1987; McKenzie et al., 1989; Naito et al., 1989).

1.3.1.2 The expression of IL-1 beta

The release of IL-1β is controlled at many levels, in order to prevent excessive amounts of active IL-1β. The translated IL-1β precursor needs to be processed by Caspase-1 into its mature form in order to be released. This depends on two distinct

extracellular signals, such as TLR agonists and ATP and the following assembly of the inflammasome (Mariathasan and Monack, 2007). However, it has also been demonstrated that IL-1β can be processed by caspase-1 independent mechanisms. Responses induced through sterile inflammation, such as fever, augmented IL-6 and hepatic acute-phase proteins, are absent in IL-1β deficient mice, but are still present in Caspase-1 deficient mice (Fantuzzi et al., 1997). Infiltrating neutrophils are very short lived and may secrete the IL-1β precursor from intracellular stores during cell death. It has been shown that IL-1β can be processed extracellularly by the common neutrophil protease proteinase-3, as well as elastase, MMP9 and granzyme A (Dinarello, 2009; Miwa et al., 1998; Schonbeck et al., 1998).

1.3.2 Interleukin-1 alpha

The literature on **IL-1α** expression is more limited than for IL-1β and much of it is very old. However, IL-1α is synthesised as a precursor of 31 kDa (proIL-1α) which is further processed by the Ca^{2+} dependent protease calpain into the mature 17 kDa form (Kobayashi et al., 1990). The 16 kDa N-terminal cleavage product, the propiece of IL-1α, is also termed IL-1α N-terminal peptide (IL-1NTP). IL-1NTP can translocate into the nucleus and is involved in RNA splicing/processing as well as activation of proinflammatory genes (Werman et al., 2004). There is no completely IL-1α deficient mouse available so far. The existing model still contains IL-1NTP, thus the effects of its absence have not been studied. IL-1α is constitutively produced at low levels in some stromal and epithelial cells. It is mainly active in its cell-associated forms, as membrane-associated molecule or as cytoplasmic precursor (proIL-1α). It is present in cells during steady-state homeostasis, although its expression is up regulated during inflammation. The latent form of calpain is activated in cells under inflammatory conditions. Epithelial cells including KCs and fibroblasts produce the IL-1α precursor

constitutively but they also contain calpain inhibitors (calpastatin) preventing the processing and release of IL-1α (Garach-Jehoshua et al., 1998; Watanabe and Kobayashi, 1994). Instead these cells store the full-length precursor of IL-1α intracellularly. Monocytes and macrophages require de novo synthesis of IL-1α. However, unlike the IL-1β precursor, the IL-1α precursor is biologically active and can signal through the IL-1RI (Mosley et al., 1987). Pro-IL-1α and the mature forms of IL-1α bind with similar affinities to the IL-1RI (Mosley et al., 1987).

It has been reported that most human cells do not readily process or secrete mature IL-1α. It is therefore not generally found in bodily fluids and is not associated with systemic effects, as IL-1β is for example. Nevertheless, there are some reports showing that IL-1α can be released from human monocytes, stimulated by activated T cells or with LPS (Bhardwaj et al., 1989; Kobayashi et al., 1990; McKenzie et al., 1989) and KCs, stimulated with IFN-γ (Pastore et al., 1998). Activated human and murine monocytes produce predominantly IL-1β, whereas the predominant form of IL-1 produced by KCs is IL-1α (Kupper et al., 1986). In contrast to human cells, mouse cells have been reported to process and secrete IL-1α more readily (Dinarello, 1996). Murine macrophages are able to release detectable amounts of IL-1α upon stimulation, whereas DCs, B- and T lymphocytes are unable to (Koide and Steinman, 1987; Koide et al., 1987; Naito et al., 1989).

1.3.3 Control of IL-1 expression

Tight control of IL-1 levels is necessary since most cells express the IL-1RI constitutively and excess IL-1 can lead to broad inflammation and tissue damage. IL-1α and β, which bind with a similar affinity to the IL-1RI (200-300 pmol/L and 500-1000 pmol/L respectively), can also bind the decoy receptor IL-1RII, which is incapable of transducing a signal into the cell and functions in reducing excessive amounts of IL-1.

IL-1β binds with an affinity of 100 pmol/L to the IL-1RII, which can also associate with IL-1RAcP when the ligand is bound, thus sequestering not only IL-1, but also the accessory protein (Dinarello, 1996). The IL-1RII is mainly expressed by cells that also produce IL-1, such as monocytes, B cells and epithelial cells. This may provide a mechanism to inhibit unwarranted autocrine activation by IL-1. KCs have been reported to express more IL-1RII than IL-1RI especially after activation (Groves et al., 1995a). IL-1RII and IL-1RAcP also exist in a soluble form, which can be released from cells and retain IL-1. A second line of controlling the effects of IL-1 is the production of IL-1RA. IL-1RA exists in four isoforms. The first isoform is mainly secreted (sIL-1RA) by monocytes, the other three are intracellular forms (icIL-1RA), which are found within KCs and other epithelial cells (Arend and Guthridge, 2000). IL-1RA will bind IL-1RI with a higher affinity (50-100 pmol/L) than IL-1α and IL-1β and therefore block their binding (Dinarello, 1996). Moreover, due to the high affinity binding of IL-1RA, IL-1AcP is not recruited and no signal is transmitted into the cell. By comparison to IL-1RI, IL-1RA as well as IL-1α bind the IL-1RII much less avidly (10-30 nmol/L) (Arend et al., 1994; Dinarello, 1996). The intracellular form of IL-1RA is constitutively expressed in KCs and can be released when the cells undergo necrosis (Arend 1998). IL-1, as well as other proinflammatory stimuli, such as LPS, induce the production and release of IL-1RA (Mee et al., 2005). However, IL-1RA needs to be in >100-fold excess to block IL-1α/β signalling (Arend et al., 1990).

1.3.4 IL-1 signalling

IL-1 signals through the IL-1RI, which changes conformation after ligand binding, to form a heterodimer with IL-1RAcP (Figure 1-3). Both IL-1RI and IL-1RAcP contain a TIR domain in their cytoplasmic part, which is shared with the TLR family. Presumably, bringing the two TIR domains into close proximity, leads to

recruitment of intracellular TIR domain containing adaptors MyD88, TIR-domain-contatining adapter-inducing interferon-beta (TRIF), TRIF-related adaptor molecule (TRAM), Toll-Interleukin-1 receptor domain-containing adaptor protein (TIRAP)/ MyD88 adaptor-like (MAL) or sterile α- and armadillo-motif-containing protein (SARM) (O'Neill and Bowie, 2007). MyD88 is crucial for the signalling via IL-1RI, IL-18Rα, IL-33Rα/ST2 and all TLRs, except TLR3, which involves TRIF. Via its death domain MyD88 can interact with members of the IL-1R-associated kinase (IRAK) family (Lin et al., 2010). Subsequently, the signal is transferred through TNF-receptor-associated factor (TRAF)6 to activate MAPKs such as p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), leading to activation of the transcription factor AP-1. Furthermore, the transcription factors NFκB and several IRFs become activated and translocate into the nucleus to influence gene expression. TLR 3 activation leads to association with TRIF, which can also be recruited to TLR4 via TRAM (Yamamoto et al., 2003), mediating the MyD88-independent arm of the TLR4 signalling cascade (Figure 1-3). The adaptor protein MAL was demonstrated to play a crucial role in MyD88-dependent signalling after TLR2 and TLR4 ligation (Yamamoto et al., 2002). However, SARM has been suggested to be a negative regulator of TRIF (Carty et al., 2006).

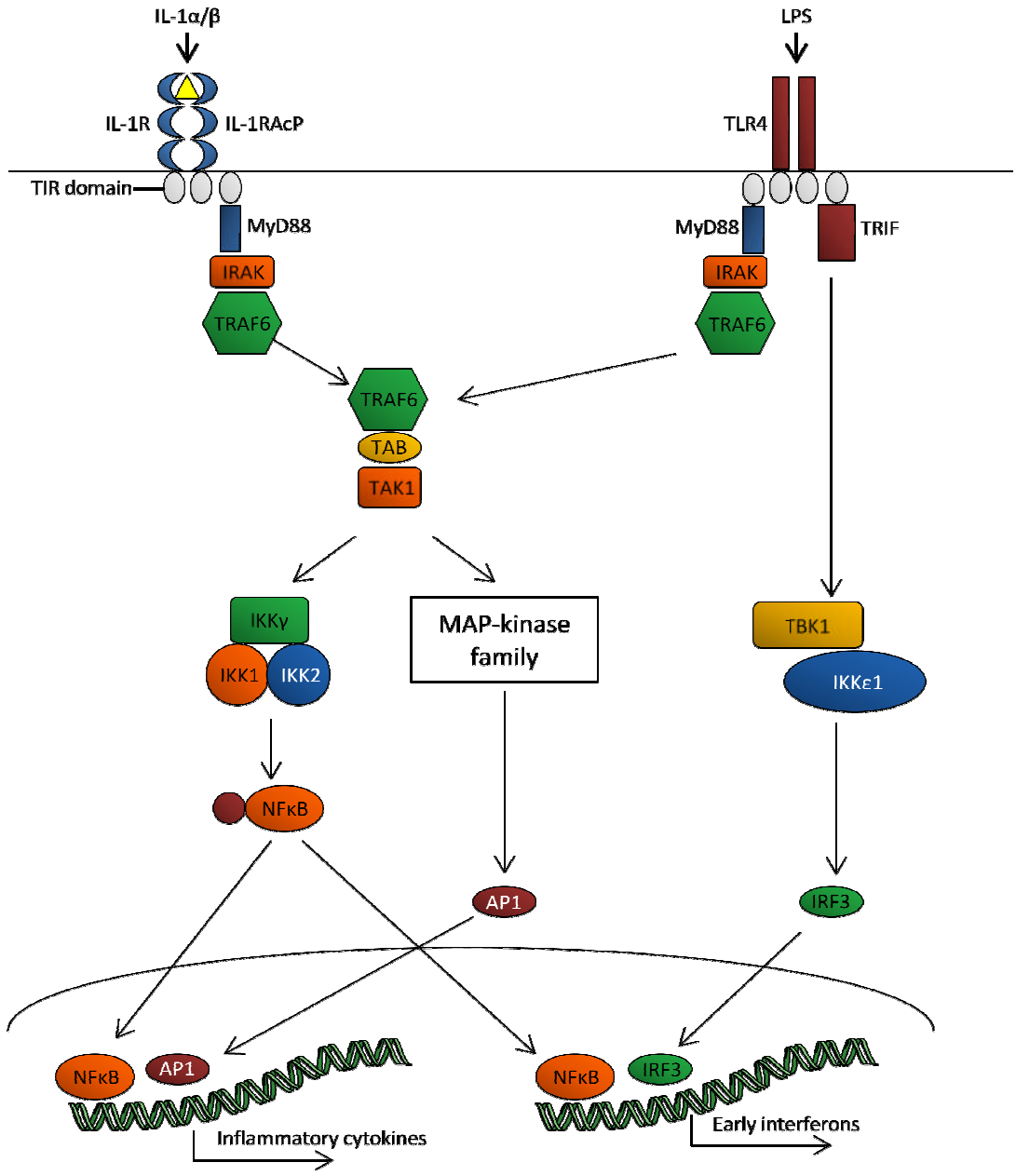


Figure 1-3: Main molecules involved in IL-1/TLR signalling.

Most TLRs and the IL-1R share the MyD88 dependent signalling pathway. IL-1 ligation leads to the formation of a heterodimer between IL-1R and IL-1RAcP. MyD88 is recruited to the TIR domains of the IL-1R/TLRs and can subsequently recruit IRAK to the active receptor complex. This leads to activation of a pathway involving many molecules, such as TRAF6, TAB1/2 and TAK1, resulting in the phosphorylation of different downstream regulatory kinases and in the nuclear translocation of NFκB and AP-1. TLR4 can additionally recruit the adaptor protein TRIF, resulting in the translocation of IRF3 and the production of early interferons.

1.3.5 IL-1 alpha in the skin

The amount of prestored IL-1α within human KCs is very considerable. 1 g of human stratum corneum has been reported to contain nearly 10^6 units of IL-1 activity (Gahring et al., 1985). Kupper et al., reports that 10 litres of the supernatant from LPS-stimulated human monocytes would contain comparable amounts (Kupper et al., 1986). It is believed that whenever KCs are damaged by UVB light, chemicals or microbial infections, they release the preformed IL-1, which is sufficient to induce a cutaneous immune response (Janssens et al., 2009; Kupper, 1990; Lee et al., 1997; Shankar et al., 1996). Release of KC-derived IL-1α induces the release of proinflammatory cytokines and upregulation of endothelial adhesion molecules in human (Groves et al., 1992) and murine (Groves et al., 1995b) skin.

KC death in the distal layers of the epidermis is a natural part of skin physiology. However, pathological cell death is found in many skin inflammatory diseases, including psoriasis, bullous disease, lichen planus, irritant or allergic contact dermatitis or during infections.

During cell necrosis pro-IL-1α is released by dying cells and can trigger a sterile inflammatory response by activating mainly non-bone-marrow-derived cells resulting in the infiltration of neutrophils (Chen et al., 2007; Cohen et al., 2010; Eigenbrod et al., 2008). IL-1α released by necrotic KCs can activate bystander KCs to produce proinflammatory cytokines and chemokines like TNF-α, IL-6, IL-8 and CCL-20 which potentiate the immune response (Bashir et al., 2009; Gahring et al., 1984; Kupper et al., 1989; Larsen et al., 1989; Spiekstra et al., 2005). Furthermore IL-1α stimulation leads to the synthesis of epidermal lipids, augmented proliferation and migration of the KCs and fibroblast proliferation (Gyulai et al., 1994; Sauder et al., 1989), therefore playing an important part in wound healing (Jung et al., 2011; McKenzie and Sauder, 1990).

Stimulation of underlying fibroblasts by KC-derived IL-1α plays an important role in skin homeostasis and wound healing (Chong et al., 2009; Lee et al., 2009; Newby et al., 2000). The release of pro-IL-1α is specific to necrotic cells, as apoptotic cells retain IL-1α by chromatin binding (Cohen et al., 2010).

1.3.6 IL-1 in CHS

The role of IL-1β during CHS responses has been studied intensively over the past years (Cumberbatch et al., 1997, 1998, 1999; Enk et al., 1993; Kimber et al., 2000; Wang et al., 1996). In IL-1β$^{-/-}$ or Caspase-1$^{-/-}$ mice the LC migration and the CHS response were attenuated following skin painting with contact sensitisers (Antonopoulos et al., 2001; Shornick et al., 1996; Sutterwala et al., 2006). Using ASC- and NALP3-deficient mice it was suggested that the inflammasome is only needed throughout the sensitisation phase of CHS (Sutterwala et al., 2006). Furthermore, local injection of IL-1RA leads to attenuated CHS responses (Kondo et al., 1995). However, the role of IL-1α in CHS has not been completely elucidated yet and is also not as well studied as the impact of IL-1β. Increased amounts of IL-1 have been reported in the epidermis during allergic patch test reactions (Larsen et al., 1988), and epidermal cultures from mice painted with TNCB showed an augmented expression of IL-1α 2h after hapten painting, and protein was found 4h after chemical exposure (Enk and Katz, 1992). It is most likely that the IL-1α found in the epidermis is released by KCs (Enk and Katz, 1992). Injection of both IL-1α and IL-1β intradermally into mice leads to a reduction of LC numbers in the epidermis and an increase of DCs in the draining lymph nodes (Cumberbatch et al., 2002). LC migration after exposure to the irritant SLS was abrogated with anti-IL-1α antibodies, but not with anti-IL-1β antibodies. Vice versa, LC migration after oxazolone exposure was attenuated after anti-IL-1β treatment, but not

after anti-IL-1α administration (Cumberbatch et al., 2002). IL-1α might also play a direct role in activation of trinitrophenol (TNP)-specific T cells (Nakae et al., 2001).

The relative importance of IL-1α and IL-1β in CHS still remain unclear. In one study, low dose TNCB-induced CHS was suppressed in IL-1β$^{-/-}$, whereas the dependency on IL-1β was reduced at high doses of TNCB (Shornick et al., 1996). This might suggest that IL-1α can compensate for IL-1β deficiency at high sensitiser doses. Higher doses of sensitisers are likely to also have higher irritant properties, therefore leading to a release of IL-1α from damaged KCs. In contrast, Nakae et al., found that similar levels of CHS were induced in wildtype and IL-1β$^{-/-}$ mice after low and high dose application of TNCB (Nakae et al., 2001). However, IL-1α$^{-/-}$ and IL-1α/β$^{-/-}$mice showed attenuated CHS responses after low and high doses of TNCB (Nakae et al., 2001). These experiments were done using mice with a C57BL/6J genetic background and the authors report that the effect of the IL-1α deficiency was reduced when using BALB/c mice (Nakae et al., 2001). Thus, the genetic background might have considerable influence on the role of IL-1 in the skin.

1.3.7 Other members of the IL-1 family in CHS

Pro-IL-18 is constitutively expressed by DCs, macrophages and epithelial cells, but needs to be cleaved by caspase-1 to become activated. Human KCs have been shown to constitutively express IL-18. However, because KCs lack caspase-1, IL-18 is not processed and is stored in its inactive form (Mee et al., 2000). IL-18 might also play a role in LC migration from the skin into the lymph nodes during CHS reactions (Cumberbatch et al., 2001). IL-33 is a proinflammatory cytokine that is mainly expressed by non-haematopoietic cells and can be released from damaged cells (Cayrol and Girard, 2009). IL-33 is thought to be inactivated by the apoptosis related caspases 1 and 3 (Cayrol and Girard, 2009). It can also interact with heterochromatin, functioning

as a transcriptional repressor. Its´ receptor ST2 is highly expressed on Th2 cells and might play a role in its´ activation.

1.4 Dendritic cells

DCs are the sentinels of the immune system, providing a crucial link between innate and adaptive immune responses. They reside in different tissues and sample antigens, which can be internally processed and presented on MHC class I or MHC class II molecules. The release of danger signals in these different microenvironments results in maturation of the DCs, which is shown by upregulation of activation/costimulatory markers and migration to the draining lymph nodes or the spleen. DCs are professional APCs and are thus able to activate and modulate naive and memory $CD4^+$ and $CD8^+$ T cell responses of T cells bearing the cognate TCR. DCs have also been implicated in peripheral T cell tolerance towards self-antigens (Albert et al., 2001; Brocker et al., 1997). In this brief survey, we focus on four limited areas within this enormous field. They are the classification of the major DC subsets, the major *in vitro* models used for the study of human DC function; the response of DC to cell death; and the specific role of DC/LC in CHS.

1.4.1 Dendritic cell subsets

According to Shortman and Naik, DCs can be classified into **conventional DCs** (cDCs) (also referred to as myeloid DC, mDC), which already show a dendritic morphology and exhibit DC functions in a steady state, and **precursor DCs** (pre-DCs), which do not have an immediate dendritic shape and DC function, but have the capacity to develop into DCs (Shortman and Naik, 2007). Monocytes and the type I interferon-producing plasmacytoid (pDCs) are examples of pre-DCs (Shortman and Naik, 2007).

Under inflammatory conditions monocytes can give rise to **Tip-DCs**, which produce inflammatory mediators, including TNF-α, nitric oxide and reactive oxygen species and might play an important role during bacterial infections (Serbina et al., 2003). Recently, it was discovered that monocytes can also give rise to **monocyte-derived DCs** (Mo-DCs), which develop in mice after administration of LPS or gram-negative bacteria (Cheong et al., 2010). **pDCs** are specialised in recognising viral nucleic acids via TLR 7 and TLR9. They are efficient producers of type I IFN, which acts to block viral replication and activates several types of innate and adaptive immune cells (Colonna et al., 2004; Siegal et al., 1999). Activated pDCs can also induce antigen-specific T cell responses (Colonna et al., 2004; Di Pucchio et al., 2008).

Conventional DCs (cDCs) subdivide into lymphoid-tissue-resident DCs and non-lymphoid DCs (Shortman and Naik, 2007). The cDC population is characterised by the lack of other lineage specific markers and a high expression of MHC class II (MacDonald et al., 2002). DCs are short lived and therefore need to be replenished by blood-borne precursors continuously. Development of DCs progresses from the common myeloid progenitor (CMP) to the macrophage and DC precursor (MDP), which can give rise to monocytes and the common DC precursor (CDP) (Liu et al., 2009). CMPs, MDPs and CDPs are all found in the bone-marrow, whereas precursors of conventional DCs (pre-cDCs), which originate from CDPs, can additionally be found in blood, spleen and lymph nodes (Arce et al., 2009; Liu et al., 2009). Pre-cDCs develop predominantly into cDCs (Liu et al., 2009), which stay in the lymphoid tissue or migrate into the periphery. CDPs can also give rise to pDCs (Liu et al., 2009). Both, lymphoid as well as myeloid progenitors, can give rise to DCs (Chicha et al., 2004; Traver et al., 2000). However, CMPs are more abundant than CLPs and thus are believed to be the major source of DCs (Chicha et al., 2004; Traver et al., 2000).

Lymphoid-tissue-resident DCs include most DCs in the spleen and the thymus, though there are also lymphoid-tissue resident DCs in the lymph nodes. Lymphoid-tissue-resident DCs do not migrate but stay in the lymphoid tissue. They show an immature phenotype and collect and present antigen in the lymphoid organ itself (Wilson et al., 2003). Two subsets of cDC were originally defined in mouse spleen: the $CD8\alpha^{+}DEC205^{+}$ subset, which plays an important role during viral infections and the uptake of dying cells and the $CD8\alpha^{-}33D1^{+}$ DC, which are specialised in presenting antigens on MHC class II (Dudziak et al., 2007). The latter can be further subdivided into $CD4^{+}$ and $CD4^{-}$ cells. There has been a lot of interest in the $CD8\alpha^{+}DEC205^{+}$ DCs as they can cross-present exogenous antigens on MHC class I. This mechanism enables DCs to induce $CD8^{+}$ T cell responses against viruses that do not infect APCs as well as maintain peripheral tolerance to peptides that are not expressed by DCs (Shortman and Liu, 2002). The lymphoid-tissue-resident DCs in humans are much less well studied than in mice, as they are not as easily accessible. Three distinct subsets of $Lin^{-}HLA\text{-}DR^{+}$ cells have been identified from human blood, which can be identified by the expression of CD1c, BDCA-3 and CD123 (MacDonald et al., 2002; Ueno et al., 2011). The $CD11c^{-}CD123^{hi}$ subset represents pDCs. The $CD1c^{+}$ (BDCA-1) subset is the major population of the $CD11c^{+}$ DC, whereas less than 5% of blood DC are $CD11c^{+}BDCA\text{-}3^{+}$. Recent reports suggest that the $CD11c^{+}BDCA\text{-}3^{+}$ cells might be the human equivalent of the mouse $CD8\alpha^{+}$ DC (Bachem et al., 2010; Jongbloed et al., 2010; Poulin et al., 2010). Both express CLEC-9A (DNGR-1), which has been implicated in the recognition of necrotic cells and DC activation (Sancho et al., 2009; Schreibelt et al., 2012). However, blood $CD1c^{+}$ DCs as well as LCs can also cross-present exogenous antigens (Klechevsky et al., 2010; Ueno et al., 2011). Immature **migratory DCs** are located in the peripheral tissues such as the skin, lung or gut, where they take up any incoming antigen, migrate back to the lymphoid tissue and present it to T lymphocytes. In mice at

least two subsets of DCs are found in non-lymphoid tissue: $CD103^+CD11b^{lo/-}$ and $CD103^-CD11b^{hi}$ DCs (Ginhoux et al., 2009). The $CD103^+CD11b^{lo/-}$ subset is thought to migrate to the lymph nodes and has been suggested to be the equivalent of the splenic $CD8\alpha^+$ DC, as it efficiently cross-presents cell-associated antigens (Bedoui et al., 2009; Edelson et al., 2010; Ginhoux et al., 2009). The lamina propia contains an additional subset of non-lymphoid DC: the $CD103^-CD11b^+CX3CR1^+$ cells, which develop from monocytes (Bogunovic et al., 2009; Varol et al., 2009). In the **skin** of mice, three distinct subsets of DCs can be found: **LCs** in the epidermis and two kinds of cDC populations in the dermis (Bursch et al., 2007; Ginhoux et al., 2007; Poulin et al., 2007). The majority of **dermal DCs** are $CD11c^+$ and $CD11b^+$. Another recently described subset can be identified as $CD103^+langerin^+CD11b^{lo}$ (Bursch et al., 2007; Ginhoux et al., 2007; Nagao et al., 2009; Poulin et al., 2007). In human skin, only two kinds of DCs can be found; interstitial DCs or dermal DCs (dDCs) which express DC-SIGN, CD11c and CD14 reside in the dermis, whereas Langerhans cells (LCs), which express CD1a, E-cadherin and Langerin, are found in the epidermis. An equivalent to the $CD103^+langerin^+CD11b^{lo}$ dermal DCs has not been identified thus far, however $CD103^+$ DCs have been found in human mesenteric lymph nodes (Jaensson et al., 2008). The expression of the C-type lectin CD207 (Langerin) is a hallmark of LCs in both human and mice. It can induce the so called Birbeck granules and seems to function as a nonconventional receptor for antigen capture (Valladeau et al., 2000). However, langerin is not exclusively expressed by LCs. $CD103^+$ DCs in the dermis, lung, kidney and liver of mice and human gut, lung and kidney cells also show langerin expression (Ginhoux et al., 2009; Merad et al., 2008). During steady-state and certain inflammatory conditions the radioresistant LCs self-renew *in situ* and are therefore not dependent on bone marrow-derived precursors (Collin et al., 2006; Kanitakis et al., 2004; Merad et al., 2002). It has been suggested that LC precursors enter the skin before

birth in a single wave and that the LC pool is maintained by proliferating, differentiated LCs (Chorro et al., 2009). However, during inflammation circulating Gr-1^{hi} monocytes can give rise to LCs (Ginhoux et al., 2006).

Originally, LCs were thought to be the main sentinels in cutaneous immune responses by inducing antigen-specific T cell responses (Reis e Sousa et al., 1993; Romani et al., 1989; Schuler and Steinman, 1985). However, this idea was challenged when Allen et al., found that lymph node-resident CD8α^+ DCs were crucial for priming HSV-specific $CD8^+$ T cell responses instead of LCs (Allan et al., 2003). It was therefore suggested that dDCs and LCs might function in sampling antigen in the epidermis and transferring it to resident CD8α^+ DCs upon arrival in the lymph nodes. However, dDCs might be the main migratory DC to cargo antigen in the early phase of a dermal Herpes simplex virus (HSV) infection as LCs migration to the lymph nodes peaks later on, at 72-96 hours (Allan et al., 2006). During the second phase of an HSV infection the recently identified dermal $CD103^+$langerin$^+$ DCs have been shown to be the main inducers of cytotoxic T cell responses by cross-presenting HSV-derived peptides (Bedoui et al., 2009). LCs also fail to induce cytotoxic T cell responses against other cytolytic viruses, such as vaccinia and influenza (Belz et al., 2004; He et al., 2006), but they are able to induce cytotoxic T lymphocyte (CTL) responses after lentiviral infection (He et al., 2006). This might suggest that cytopathic effects of some viruses may inhibit LCs induced CTL responses. However, bystander DCs might be able to pick up antigen from dying cells and cross-present it to $CD8^+$ T cells. In a similar fashion to mice, it was shown in humans that apoptosis induced by HSV compromises LC function in the skin (Bosnjak et al., 2005). However, most studies have been executed *in vitro* suggesting a role for LCs in Th1 and CTL responses, whereas dDC were mainly found to initiate follicular Th responses as well as mediating B cell Ig class

switching (Klechevsky et al., 2008). In a mouse model of graft-versus-host disease it was recently suggested that LCs license skin infiltrating $CD8^+$ T cells to develop into fully functional effector cells that can induce tissue injury. However, they were not involved in the priming or recruitment of the T cells (Bennett et al., 2011). The role of DC and LC in CHS is discussed in further detail below.

1.4.2 Experimental model: monocyte-derived dendritic cells

The study of human DCs is not an easy task as they are not readily available. It is possible to isolate human DCs from tissues, such as tonsils. However the procedures are very time consuming and large amounts of tissue is needed, which is not always readily available (Brennan et al., 1987; King and Katz, 1989). Though human blood is more easily accessible, DCs are very rare and they show a heterogenous expression of surface markers due to their activation status and linage commitment. Blood DCs are comprised of immature and mature DC and can be subdivided into at least four groups (MacDonald et al., 2002; Ueno et al., 2007). The low numbers are often not sufficient for experimental studies, making it a very challenging task to work with primary human DCs. Therefore, the observation that monocytes are able to differentiate into DCs in the presence of GM-CSF and IL-4 *in vitro* revolutionised the work on human DCs (Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996). It is still debated if monocytes can give rise to DCs *in vivo*, leading to questions regarding the physiological significance of the monocyte-derived DC model. It is well established now that under steady state conditions monocytes develop from monocyte precursors, while cDCs develop from pre-DCs. However, under inflammatory conditions monocytes can directly give rise to Tip-DCs (Serbina et al., 2003) (see chapter 1.4.1). Furthermore, in a mouse model it was recently discovered that LPS-containing bacteria can stimulate monocytes to differentiate into DC-$SIGN^+$/MMR^+ DCs *in vivo* (Cheong et al., 2010). DC-

SIGN/CD209 and MMR/CD206 are C-type lectins which are also found on human monocyte-derived DCs, obtained by culture in GM-CSF and IL-4. Mo-DCs differentiated in response to LPS showed the typical DC morphology and were found to be similar to lymph-node resident $CD8^{-}DEC205^{-}$ cells in their expression of numerous surface markers (Cheong et al., 2010). However, they showed lower expression of CD11c and did not express CD135/Flt3 (Cheong et al., 2010). The Mo-DCs localised to T cell areas in the lymph nodes and showed the ability to present antigen and stimulate mixed leukocyte reactions (MLRs) (Cheong et al., 2010). Moreover, they were as efficient as or even more effective than, $CD8\alpha^{+}$ DCs in cross-presentation (Cheong et al., 2010). Therefore, Mo-DCs are likely to play a role *in vivo* and show similarities to other DC subsets; however they are also likely to have properties distinct to cDCs. A similar subset has not been identified in humans thus far. Nevertheless, human $CD14^{+}$ monocytes undergoing reverse transmigration (a process involved in transendothelial trafficking) were shown to differentiate into DC-like cells (Randolph et al., 1998).

Human Mo-DCs are a widely used model for DCs as they display many characteristics of cDCs (Grassi et al., 1998). They are non-adherent cells with processes that can extend and retract from the cell body. Immature Mo-DCs do not express monocyte markers such as CD14 and FcγRI, however they do express CD1, CD11c, FcγRII, DC-SIGN, MHC class I and II and costimulatory molecules CD40, CD54/ICAM-1, CD80/B7-1 and CD86/B7-2 (Geijtenbeek et al., 2000c; Sallusto and Lanzavecchia, 1994; Woodhead et al., 1998). Upon stimulation they upregulate the expression of MHC and costimulatory molecules, as well as the DC maturation marker CD83 (Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996). Moreover, they have the ability to process and present antigen to naive T cells and influence their functionality (Sallusto and Lanzavecchia, 1994).

Monocytes can also differentiate into LCs. *In vitro* studies showed that human dermal-resident $CD14^+$ cells, as well as monocytes derived from peripheral blood, can differentiate into LC-like cells in the presence of GM-CSF, IL-4 and TGF-β (Geissmann et al., 1998; Larregina et al., 2001). In a murine *in vivo* model, Gr-1^{hi} monocytes were identified as direct precursors of LCs during skin injury (Ginhoux et al., 2006). TGF-β, which is synthesised at high levels by KCs, has been suggested to play a crucial role during LC development, as TGF-β knock-out mice are devoid of LCs (Borkowski et al., 1996). The role of TGF-β in LC differentiation *in vivo* remains unclear, although it has been suggested that autocrine TGF-β might be required for LCs to develop (Kaplan et al., 2007).

1.4.3 Activation of Dendritic cells

As mentioned in Chapter 1.2.1 PAMPs and DAMPs can be recognised by germline encoded PRR. Most TLRs, NLRs, RLRs and CLRs are expressed by DCs, as they play a crucial role in sensing danger/pathogens to alert the adaptive immune system. The interaction of DC with a wide variety of pathogens has received an enormous amount of interest, but will not be considered here. Instead, we focus on the ability of DCs to recognise dying cells.

Both apoptotic and necrotic cells can be taken up by immature DCs (Gallucci et al., 1999), but only necrotic cells induce maturation of immunostimulatory DCs (Newton et al., 2003; Sauter et al., 2000). Furthermore CLEC9A has been identified as a receptor in DCs sensing necrotic cells, although its ligand has not been identified yet (Sancho et al., 2009). The uptake of apoptotic cells, in contrast, is believed to be important for the maintenance of peripheral tolerance, as reviewed in (Steinman and Nussenzweig, 2002). Huang et al. identified a DC subset that constitutively carries apoptotic bodies derived from the intestinal epithelium to T cell areas in the mesenteric

lymph nodes *in vivo* (Huang et al., 2000). These immature self-antigen carrying DCs are either believed to tolerise T cells directly because of a lack of costimulatory molecules or transfer the tissue-derived peptides to tolerogenic lymphoid-tissue resident DCs (Steinman et al., 2000). On the other hand, macrophages have been found to modulate immune responses after phagocytosis of apoptotic cells by inducing immunosuppressive and anti-inflammatory effects (Fadok et al., 1998; Gao et al., 1998; Voll et al., 1997). It has been shown recently that apoptotic cells can either actively promote inflammation or induce anti-inflammatory immune responses, depending on whether the dying cell is infected or not (Torchinsky et al., 2009). Ingestion of neutrophils infected with bacteria by DCs led to the differentiation of Th17 cells, whereas the ingestion of uninfected neutrophils by DCs led to TGF-β and IL-10 production by DCs favouring the induction of regulatory T cells (Torchinsky et al., 2009).

1.4.4 The role of dendritic cells and Langerhans cells in Contact Hypersensitivity

DCs are one of the key players in ACD as they are sentinels in the skin, which can recognise hapten modified protein as well as elicit an adaptive immune response. Both dDC and LC have been shown in CHS models to take up haptenated protein and migrate to the lymph nodes (Hill et al., 1990; Macatonia et al., 1987). It has been well documented that following skin painting of mice with haptens LCs exit the skin and migrate to the lymph nodes. This egress is dependent on TNF-α, produced by KC, as well as IL-1β produced by LCs (Cumberbatch et al., 1997; Enk et al., 1993). IL-1β is believed firstly to act in an autocrine manner on the LCs themselves and secondly to stimulate KCs to produce TNF-α (Cumberbatch et al., 1998, 1999; Wang et al., 1996). The release of IL-1β during CHS might be dependent on extracellular ATP and the inflammasome as mice lacking the purinergic receptor $P2X_7$ are resistant to CHS

(Weber et al., 2010). IL-1β and TNF-α act in concert to stimulate LCs to upregulate the expression of costimulatory molecules, such as ICAM-1, CD80/CD86 and MHC class II. Attenuated expression of E-cadherin and augmented expression of α4 and β1 integrins and MMP-9 facilitates detachment from the surrounding KCs and migration out of the epidermis. Expression of the chemokine receptor CCR7 enables homing to the paracortical region of the draining lymph nodes. Moreover, IL-18 has been shown to play a role in LC migration following Oxazolone administration (Cumberbatch et al., 2001; Wang et al., 2002). IL-18 seems to act upstream of IL-1β and TNF-α (Cumberbatch et al., 2001). LC migration might be regulated by IL-10 and IL-4 (Takayama et al., 1999; Wang et al., 1999a)

Nevertheless the contribution of LCs towards CHS reactions remains controversial. Three distinct LC ablation models have been developed to give further insight into the role of LCs during CHS, but all three of them gave different results. The first two models used a gene targeted mouse that expresses a diphtheria toxin receptor under the control of the Langerin promotor. In the first model CHS was reduced after depletion of LCs (Bennett et al., 2005). In the second model CHS was unaffected by the removal of LCs (Kissenpfennig et al., 2005). However, in the third model, where mice lack LCs from birth, the CHS response was increased (Kaplan et al., 2005). Other mechanisms might substitute the constitutive absence of the LCs. In the first and second model the diphtheria toxin was expressed under the control of the langerin promotor, leading not only to the depletion of LCs, but also of the dermal $CD103^{+}$langerin^{+} subset. The dermal langerin^{+} cells reappear more quickly than the epidermal LCs. Therefore, by choosing the right time window of toxin administration and hapten painting the CHS reaction can be studied either in the absence of both langerin^{+} subsets or only in the absence of LCs. Such experiments suggest that dermal $CD103^{+}$ langerin^{+} DCs are

sufficient to induce CHS responses as CHS reactions were unaltered after LC depletion, but were attenuated when both langerin^{+} subsets were absent (Bursch et al., 2007; Wang et al., 2008). However, when CHS responses were assessed in Batf3$^{-/-}$ mice, which lack the lymphoid resident CD8α^{+} DC as well as the CD103^{+}CD11b^{-} migratory DC, including the CD103^{+}langerin^{+} dDC, CHS responses were normal, suggesting no absolute requirement of the langerin^{+} dDC to induce CHS (Edelson et al., 2010). Furthermore, the hapten dose, and therefore the number of DCs that have access to the hapten influence the CHS response (Noordegraaf et al., 2010). The exact role of the three skin DC populations during CHS has not been completely understood to this date; however there seems to be a lot of redundancy between the different subtypes.

Several TLRs have been implied as being involved during CHS reactions. MyD88-deficient mice failed to show a CHS response after DNFB painting (Klekotka et al., 2010), showing a crucial role for IL-1R/TLR signalling. In response to TNCB, oxazolone and FITC TLR2 and TLR4 signalling is indirectly triggered (Martin et al., 2008). TLR2 and TLR4 double knock-out mice were unable to mount a CHS reaction in response to these chemicals (Martin et al., 2008). It has been suggested that endogenous TLR2/TLR4 ligands formed in the skin rather than microbial products play a role in CHS induction (Martin et al., 2008). However, in humans TLR4 has been shown to directly interact with Ni^{2+} and transfer Ni^{2+} sensitivity (Schmidt et al., 2010).

1.5 T lymphocytes

1.5.1 Primary T cell responses

Lymphocytes mediate adaptive immune responses which allow the body to tailor its´ defence specifically to the invading pathogen, adapt to possible evading strategies of the pathogen and provide memory of past encounters. Primary T cell responses are

mediated by naive T cells, which after maturation in the thymus patrol the blood and lymphoid tissues for foreign antigens. T cells recognise peptides in the context of MHC molecules, with $CD8^+$ T cells responding to peptides presented on MHC class I and CD4 facilitating the recognition of peptides presented on MHC class II molecules. Professional APCs, such as DCs or LCs can initiate naive T cell responses. When a TCR binds its cognate antigen (peptide-MHC complex) an immunological synapse is formed between the T cell and the DC enabling the T cell to become activated. Two to four hours of DC-T cell contact have been shown to be sufficient to activate naive $CD8^+$ T cells (van Stipdonk et al., 2001), whereas at least six hours are required to induce clonal expansion of naive $CD4^+$ T cells (Celli et al., 2007). TCR ligation in the absence of costimulatory signals provided by the APC renders T cells anergic, a process called peripheral tolerance. The DC surface molecules CD80 (B7.1) and CD86 (B7.2) need to bind CD28 on T cells in order to provide costimulation. The immunological synapse is further stabilised by interaction between ICAM-1/2 on DCs and LFA-1 on T cells. T cell activation results in the upregulation of several other molecules, such as CD40L, OX40 or 4-IBB, which bind their counterparts on DC, creating a costimulatory feedback loop. Upregulation of the T cell markers CTLA-4, which binds CD80/CD86 and PD-1, which binds PD-L1/PD-L2, terminates the DC-T cell interaction (Butte et al., 2007; Walunas et al., 1994).

Successful activation of naive T cells induces proliferation (clonal expansion), increasing the numbers of a T cell clone with a certain specificity by more than 1000-fold (Blattman et al., 2002). Most of the T cells become effector T cells and home back to the site of inflammation, whereas only a small percentage develops into memory cells.

1.5.2 Effector T cells

$CD8^+$ effector T cells recognise endogenous antigen presented on MHC class I molecules. Their main role is to clear virus infected cells, through specific killing mechanisms such as Fas-FasL interaction or Perforin/Granzyme release in order to prevent further virus spread (Bhardwaj et al., 1994). $CD8^+$ T cells are therefore often referred to as cytotoxic T lymphocytes (CTL).

$CD4^+$ effector T cells mainly function in activating or helping other immune cells to exert their roles. That is why they are often called T helper (Th) cells. Th cells can be functionally subdivided into several populations based on their cytokine profiles.

Th1 and Th2 cells were the first to be identified (Mosmann et al., 1986). Stimulation of naive CD4 T cells in the presence of IL-12 skews their development towards a **Th1** phenotype (Hsieh et al., 1993). The transcription factor T-bet has been shown to play a crucial role during that process (Lighvani et al., 2001). CD4 T cells of the Th1 subtype are characterised by the production of IFN-γ and IL-2. IFN-γ can activate non-immune cells such as KCs and fibroblasts (Clark et al., 1989) as well as cells of the innate immune system such as DC, monocytes and macrophages. In mononuclear phagocytic cells it has been shown that IFN-γ priming makes these cells more sensitive to PAMP induced activation (Frasca et al., 2008; Sun and Ding, 2006; Zhao et al., 2006). Th1 cells play a crucial role in the control of intracellular pathogens by activating macrophages and CTL responses. Macrophages need Th1 help to efficiently phagocytose and kill intracellular pathogens, such as TB. CTL responses are indirectly promoted by Th1 cells by licensing DC to activate them (Bennett et al., 1998; Cella et al., 1996; Ridge et al., 1998; Schoenberger et al., 1998). IL-12, produced by licensed DC, not only enhances cytotoxic properties of $CD8^+$ T cells, but also supports further IFN-γ release, leading to a paracrine feedback loop (Hsieh et al., 1993; Manetti

et al., 1993). It has been shown in CD4-deficient mice that primary CTL responses can be induced; however secondary responses were diminished (Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003).

Th2 cells develop in the presence of IL-4 and IL-2 (Le Gros et al., 1990; Swain et al., 1990). They produce a different set of cytokines, including IL-4, IL-5 and IL-13. The transcription factor GATA-3 has been identified as the master regulator of this cell type (Ho et al., 2009). The main role of Th2 cells is to promote the humoral host defence against extracellular parasites, such as helminths. Moreover, Th2 cells play a pathogenic role during allergic diseases, such as asthma or atopic dermatitis, as reviewed in (Liew, 2002). IL-4, released by Th2 cells, encourages antibody production by B cells, especially IgE, which binds FCεRI on basophils and mastcells (Shimoda et al., 1996). Activation of these cells results in the release of histamines and several cytokines (Kawakami and Galli, 2002).

More recently **Th17** cells have been described, which produce IL-17A, IL-17F, IL-21 and IL-22 (Harrington et al., 2005). In mice Th17 cells can be differentiated *in vitro* from naive CD4 T cells in the presence of TGF-β and IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). IL-1β and IL-23 are required to maintain the cells. In humans it was reported that IL-1β, IL-6, IL-23 and low doses of TGF-β can induce differentiation of naive CD4 T cells into Th17 cells (Acosta-Rodriguez et al., 2007; Volpe et al., 2008; Wilson et al., 2007). However, IL-4 and IFN-γ suppress the development of Th17 cells. Moreover, the transcription factor RORγT/RORC2 (mice/humans) is the master regulator for the development of Th17 cells (Ivanov et al., 2006; Manel et al., 2008). Th17 cells play an important role in maintaining the barrier immunity at mucosal surfaces, such as the gut or lung (Khader et al., 2009). However, they are also found in the skin. They are crucial in protecting the host against invading

pathogens, especially extracellular bacteria and fungi, but are additionally involved in mediating autoimmune diseases, such as Crohn's disease, multiple sclerosis, psoriasis or atopic dermatitis (Khader et al., 2009; Langrish et al., 2005).

Another subset of $CD4^+$ T cells, the **regulatory T (Treg) cells**, show anti-inflammatory properties and are able to suppress immune responses. These cells are characterised by the expression of the transcription factor Forkhead box P3 (FoxP3), the constitutive expression of the IL-2 receptor-α (IL-2Rα) chain (CD25) and the production of IL-10 and TGF-β (Bennett et al., 2001; Brunkow et al., 2001; Fontenot and Rudensky, 2005; Sakaguchi et al., 1995). Two subtypes of Tregs have been described, natural Treg cells and inducible Treg cells. Natural Treg cells develop in the thymus from $CD4^+$ T cell precursors, whereas inducible Treg cells develop in the periphery from naive $CD4^+CD25^-$ T cells, both in the presence of IL-2 and TGF-β (Fontenot et al., 2005; Kretschmer et al., 2005; Marie et al., 2005). Treg cells play an important role in immune homeostasis and tolerance (Vignali et al., 2008).

Effector T cells are usually short-lived; some will however develop into antigen-experienced memory cells providing long-term protection.

1.5.3 Memory T cell response

Memory T cells patrol lymphoid as well as non-lymphoid tissues and respond to re-infection more rapidly than effector T cells during a primary infection. This pronounced response may be due to higher numbers of T cells carrying TCRs with the same specificity. Furthermore, memory T cells can respond to lower antigen concentrations, a wider range of cells can act as APCs to activate them and they need less time and fewer costimulatory signals to become activated (Berard and Tough, 2002). Two types of memory T cells can be distinguished. $CD62L^+CCR7^+$ **central**

memory T cells mainly traffic between secondary lymphoid organs; $CD62L^{-}CCR7^{-}$ **effector memory T cells** migrate through peripheral tissues, ready to elicit a rapid immune response upon antigen encounter (Sallusto et al., 1999). Effector memory T cells play a crucial role in peripheral tissue surveillance as they patrol non-lymphoid tissues. High numbers of antigen-experienced cells can be found e.g. in the liver, lung, gut or the skin.

1.5.4 T cell function during contact hypersensitivity

CHS reactions are classified as DTH responses. DTH responses to topically applied or intradermally injected antigens can be used to assess whether an individual has been sensitised with that antigen before and therefore exhibits a memory immune response. The classical example is the Mantoux test, during which a mixture of *mycobacterium tuberculosis*-derived peptides and carbohydrates are injected intradermally into the forearm skin (Ahmed and Blose, 1983). 48 hours later the diameter of the induration is assessed as a measure for previous TB infection. Similarly, patch tests are used to measure the immune response against common airway or skin allergens.

A DTH response typically shows an early nonspecific inflammation which is found in sensitised as well as non-sensitised individuals. This unspecific response is caused by trauma and/or the antigen and is marked by infiltration of innate immune cells, such as neutrophils (Platt et al., 1983). The second, antigen-specific response only occurs in sensitised people and reaches is maximum after about 48-72 hours. The majority of immune cells found at the site of antigen administration at this time are T lymphocytes. In a classical DTH response $CD4^{+}$ T cells, mainly of the Th1 type, exceed the $CD8^{+}$ T lymphocyte numbers. However, a marked difference between CHS and classical DTH is that CHS is mainly mediated by $CD8^{+}$ T lymphocytes, which are found

at higher numbers than $CD4^+$ T cells during the antigen-specific response. The role of both $CD4^+$ and $CD8^+$ T lymphocytes during CHS reactions is discussed below.

1.5.4.1 Initiation of a T cell response during the elicitation phase of a CHS response

The conventional model of a DTH or CHS response suggests that memory T cell responses are stimulated in lymphoid tissues after migration of LCs or dermal DCs from the site of insult to the draining lymph nodes. Upon recognition of the antigen by memory T cells with the cognate TCR, activation and proliferation of the T cells is induced followed by their migration to skin.

However, memory T cells might also be directly activated in the skin. Normal skin of a healthy individual contains more than 2×10^{10} skin-resident cells (or $\sim 1 \times 10^6$ cells/cm^2), which is more than twice as many T cells than are found in the blood circulation (Clark et al., 2006). The majority of T cells present in the skin are memory cells, with equal numbers of $CD4^+$ and $CD8^+$ cells. These cells show high expression of cutaneous lymphocyte-associated (CLA) antigen (Clark et al., 2006; Kupper and Fuhlbrigge, 2004). Several cells in the skin are able to present antigens to local memory T cells, such as LCs, dDCs, macrophages, KCs and human dermal microvascular endothelial cells. It is not clear however, how activation of memory T cells in the skin contributes to CHS and if it would be sufficient to induce an immune response. A recent publication using a HSV infection model, suggested that $CD8^+$ memory T cells mainly home to the epidermis, whereas $CD4^+$ memory cells are predominantly found in the dermis (Gebhardt et al., 2011). $CD8^+$ memory T cells were not very motile and seemed to be trapped in the epidermis, as opposed to $CD4^+$ T cells, which trafficked rapidly through the dermis (Gebhardt et al., 2011).

As aforementioned, during CHS responses (against strong haptens) $CD8^+$ T lymphocytes play a predominant role in mediating the disease, whereas $CD4^+$ T cells show mainly a regulatory function. The evidence for this is (1) CHS reactions were abrogated in MHC class I deficient mice or after depletion of $CD8^+$ T cells, (2) enhanced CHS responses were found in MHC class II deficient mice and after depletion of $CD4^+$ T cells and (3) only adoptive transfer of DCs from MHC class II knock-out mice were able to transfer CHS, but not from MHC class I knock-out mice (Akiba et al., 2002; Bouloc et al., 1998; Bour et al., 1995; Gocinski and Tigelaar, 1990; Kolesaric et al., 1997; Krasteva et al., 1998; Vocanson et al., 2006; Xu et al., 1996). As mentioned previously, there are two phases of inflammation during an ACD reaction. First, a local inflammation is induced through trauma and/or the haptens, leading to production of TNF-α and IL-1β. The upregulation of E-/P-selectins and ICAM-1/VCAM-1 as well as the release of several chemokines (CCL2, CCL5, CCL20, CCL22 and/or CCL27) attracts effector T cells and mononuclear cells to the skin (Albanesi et al., 2001; Vocanson et al., 2009). Skin homing effector T cells show high expression of homing markers CLA, CCR4 and CCR6, which they acquire through contact with tissue-derived DCs and possibly mesenchymal cells: a process called imprinting (Kupper and Fuhlbrigge, 2004; Nestle et al., 2009). Upregulation of CCR10 by Vitamin D has also been suggested to play a role in skin homing of memory T cells (Sigmundsdottir and Butcher, 2008). TNF-α and IL-1β can activate KCs to release CCL27 (CTACK) that binds the receptor CCR10 (Homey et al., 2002). Most skin-infiltrating lymphocytes from psoriasis, atopic and allergic contact dermatitis patients have been shown to express CCR10, suggesting an important role for CCL27-CCR10 in skin homing of T cells during inflammation (Homey et al., 2002). Recognition of haptenated peptides by effector T cells results in the release of IFN-γ and TNF-α, as well as IL-4 and IL-17. This is followed by another wave of cytokines and chemokines, namely IL-1, IL-6,

TNF-α, GM-CSF, IL-8, CCL17 (TARC), CCL18 (PARC), CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC), produced by different skin cells, including KCs and possibly mast cells (Albanesi et al., 2001). A second wave of leukocyte infiltration (T lymphocytes, neutrophils and inflammatory macrophages) follows the release of these pro-inflammatory factors (Vocanson et al., 2009).

The pathophysiology of ACD is critically dependent on $CD8^+$ T lymphocytes and their cytotoxic effects on KCs (Akiba et al., 2002). Mice deficient in perforin and Fas/FasL pathways that facilitate the cytotoxic effects of $CD8^+$ T cells were unable to mount a CHS response against DNFB (Kehren et al., 1999). Both pathways were shown to be redundant as depletion of only one of the pathways did not abrogate CHS reactions. $CD8^+$ T lymphocytes were shown to produce IFN-γ and TNF-α or IL-17, leading to a synergistic activation of KCs (He et al., 2009). IFN-γ also results in augmented expression of FasR on the KCs, making them more susceptible to Fas mediated killing by $CD8^+$ T cells (Trautmann et al., 2000).

$CD4^+$ cells have been shown to be mainly skewed towards the Th1 type, promoting the ACD response by producing IFNγ (Wang et al., 2000), although low numbers of Th2 cells can be found as well. These seem to regulate the sensitisation and effector phase of CHS by limiting the size of the $CD8^+$ effector cell pool (Gocinski and Tigelaar, 1990; Xu et al., 1996). Moreover, Th17 cells have been suggested to play a role during ACD. This was first indicated by the finding that IL-17-deficient mice show impaired CHS responses (Nakae et al., 2002). Later, nickel-specific Th17 cells were isolated from the memory T cell pool of patients with nickel allergy, whereas these cells were not found in healthy controls (Larsen et al., 2009).

Among the $CD4^+$ T lymphocytes seems to be a regulatory subset of cells, as mice depleted or deficient in $CD4^+$ T cells showed increased CHS responses. Moreover, depletion of the $CD4^+CD25^+$ subset with an anti-CD25 mAb led to an augmented CHS response (Dubois et al., 2003; Kish et al., 2005). Regulatory $CD4^+CD25^+FoxP3^+$ T cells are often depleted with an anti-CD25 antibody. However, one has to consider that CD25 is also expressed on some activated T cells, which complicates interpretation of these experiments. Many questions regarding the role of regulatory T cells during ACD remain to be studied.

1.6 Development of an *in vitro* alternative to the local lymph node assay

ACD is a common occupational and environmental health problem. Therefore, prior to market introduction of new products or product ingredients that will contact the skin, a thorough skin sensitisation risk assessment has to be carried out in order to ensure that the product will not induce ACD. Until the mid 1990s guinea pig assays were used to identify skin sensitising chemicals. For that, during the induction phase the test compound was applied onto the skin or injected intradermally. The elicitation reaction following challenge with the same compound was assessed in order to determine if the animal had been sensitised (Buehler, 1965; Magnusson and Kligman, 1969).

1.6.1 Local lymph node assay

To date the murine local lymph node assay (LLNA) has been the most widely used assay to test the sensitisation hazard of chemicals, as reviewed in (Gerberick et al., 2000). The LLNA is a substantial refinement over the guinea pig assay, providing a quantitative measure of skin sensitisation potency using the EC3% value. Chemicals are

topically applied to the mouse ear and the T cell proliferation in the draining lymph node is measured after 48 hours. The induction phase of a CHS response is therefore used as a measure. An increase in T cell proliferation of at least three-fold over the control classifies a chemical as a sensitiser. The EC3% value is the percentage concentration of a test compound, which results in three-fold increase in T cell proliferation, providing a potency measure (Kimber et al., 2002). Chemicals can be classified as extreme, strong, moderate, weak, and non-sensitisers (Kimber et al., 2003). This method has been intensively validated and is accepted world-wide as the method of choice to identify skin sensitisers (Basketter et al., 2002).

However, researchers have been trying to find alternative *in vitro* methods to characterise the sensitising potency of chemicals in order to replace the need for animal test (e.g. LLNA) data to inform risk assessment decision-making.

1.6.2 *In vitro* alternatives

Different approaches are being investigated for the development of an *in vitro* assay for the identification and characterisation of skin sensitisers (Adler et al., 2011). *In silico* methods are aiming to predict the sensitisation hazard of a novel compound by comparing its motifs with well-characterised chemicals (sensitisers), containing the same motifs. Such approaches are referred to as Structural Activity Relationship (SAR) or Quantitative Structural Activity Relationship (QSAR) (Karlberg et al., 2008). *In vitro* peptide binding assays are being used to study the haptenation of model peptides in an attempt to predict the intrinsic sensitising potential of a chemical from its peptide reactivity (Basketter and Maxwell, 2007). Other groups are investigating the ability of haptens to penetrate the skin or activate KCs using skin equivalents or KC cell lines (Basketter and Maxwell, 2007; Emter et al., 2010).

Most research groups, though, seem to focus on the direct effects of skin sensitisers onto DCs, as they play a pivotal role in the initiation and maintenance of adaptive immune responses. The hypothesis here is that DCs can distinguish between sensitiser- and irritant-induced changes by reacting to environmental and antigenic signals in the skin and converting them in terms of surface marker expression and cytokine production to modulate T cell responses. Several approaches are being used by different groups to assess this hypothesis.

It has been reported that different haptens can directly activate cultured DCs derived from PBMCs or $CD34^+$ hematopoietic cell precursors. Activation was shown by the upregulation of maturation markers (HLA-DR, CD40, CD54, CD80, CD83, CD86 and CCR7) as well as cytokine expression (IL-6, IL-12p40, TNF-α, IL-1β) (Aiba et al., 2003; Aiba et al., 2000; Aiba et al., 1997; Arrighi et al., 2001; De Smedt et al., 2005; Tuschl et al., 2000). Nevertheless the data between groups are not always consistent. Some groups found the IL-1β mRNA expression to be increased after chemical allergen exposure of PBMC-derived LCs, but not after treatment with non-sensitising irritants (Pichowski et al., 2000; Pichowski et al., 2001; Reutter et al., 1997). Other investigators, using similar experimental designs, were not able to detect allergen-provoked increases in IL-1β expression (Aiba et al., 2000; Tuschl and Kovac, 2001). The differences between sensitisers and irritants are often subtle and especially weak sensitisers are not distinguished from irritants with the *in vitro* approaches to date. CD86 expression seems to be one of the best markers available at the moment (Aiba et al., 1997; Coutant et al., 1999; Hulette et al., 2005; Tuschl et al., 2000). Furthermore, finding the optimal concentration of the chemicals is not trivial. Higher concentrations generally lead to an augmented expression of various DC activation markers, but too high concentrations are accompanied with cytotoxicity (Hulette et al., 2005; Manome et

al., 1999). It has been suggested that some low level cell trauma may even be necessary in order for DCs to respond to haptens (Matzinger, 2002; McFadden and Basketter, 2000). Another limitation of the hematopoietic cell precursors or PBMC derived DCs is the considerable donor to donor variation in responsiveness. Populations can even be divided into responders and non-responders (Pichowski et al., 2001) making the development of standardised testing procedures difficult.

Therefore, the use of DC-like cell lines, which are also less time consuming to prepare, might provide a useful tool. Various cell lines were investigated as a potential model to predict skin sensitisation potential, including THP-1 (Miyazawa et al., 2007; Yoshida et al., 2003), KG-1 (Hulette et al., 2002; Yoshida et al., 2003), U937 (Python et al., 2007) and MUTZ-3 (Azam et al., 2006) lines. To date, the THP-1 line looks like the most promising cell line for the establishment of an *in vitro* method for skin sensitisation, detecting 6 out of 8 sensitisers via CD54 and CD86 expression (Yoshida et al., 2003). Nevertheless cytotoxicity and therefore finding the optimal concentration for each sensitiser creates a problem as it influences the CD86 and CD54 expression (Sakaguchi et al., 2009). Similarly to the primary DC-like cells, the parameters of activation examined to date have only shown relatively modest changes despite using very strong sensitisers, which questions the sensitivity of the assays.

Recently, the emphasis on development of *in vitro* assays to predict the sensitising potential of chemicals seems to be shifting towards T cell based assays. The latest advances in this area are reviewed in Martin et al. (Martin et al., 2010), and are beyond the scope of this introduction. Mathematical modelling of innate as well as adaptive immune responses might provide further insights towards the development of an *in vitro* assay (Maxwell and Mackay, 2008).

1.7 Aims

Finally, although there are a lot of descriptive *in vitro* data in terms of DC responses to haptens there is a huge gap to the immunological knowledge we have already gained from countless *in vivo* CHS-experiments in mice. *In vivo* as well as *in vitro* the data on the role of the innate immunity in CHS are limited. It is still not fully described how LCs/DCs recognise haptenated proteins and which PRRs might be involved in that process. Therefore I want to develop a more sensitive model to investigate the sensitising potential of different chemicals by limiting the chemical induced cytotoxicity of DCs. We want to achieve that by labelling a human KC cell line with different sensitisers. The remaining chemicals will be washed off before the sensitiser-labelled KCs are cocultured with DCs, thus not exposing DCs to the cytotoxic concentrations of chemical. The dying KCs could even provide "danger signals" which may enhance the sensitivity of the assay, as suggested by Basketter and Maxwell (Basketter and Maxwell, 2007). Furthermore, this two cell model will allow us to study the interaction of KCs and DCs. We also want to use this model to assess the hypothesis that DCs can recognise haptenated proteins via a PRR as well as study the mechanisms that are involved. The principal aims of my thesis are to:

1. Develop a new *in vitro* model to study the interaction of KCs and DCs in the presence of different sensitisers/irritants
2. Test the hypothesis that sensitisers can act as PAMPs and activate DCs
3. Test the hypothesis that sensitisers can induce the release of DAMPs
4. Dissect the activation of DCs by IL-1α on gene expression, protein and functional level.

2 MATERIAL AND METHODS

2.1 General tissue culture techniques

All tissue culture work was carried out in class II safety cabinets and sterile tissue culture techniques were applied. Cells were cultured in humified incubators at 37°C and 5% CO_2. All tissue culture flasks, plates and dishes were purchased from TPP (Helena Biosciences) or Nunc (Thermo Fisher Scientific).

2.1.1 Media

Roswell Park Memorial Institute (RPMI)-1640 and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen. Media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Biosera), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) to obtain complete media. Different lots of FBS had been tested for ensuring DC survival and a robust immature/mature phenotype. The optimal serum (cell survival, DC phenotype) was used throughout the study.

Serum-free AIM-V medium (Invitrogen) was used to culture HaCaT cells for immunoblotting. EpiDerm™ cultures were established in serum-free DMEM (MatTek).

2.1.2 Freezing and thawing of cells

Cells were pelleted and resuspended in 1 ml of freezing mix (10% (v/v) DMSO (Sigma Aldrich) in FBS) and transferred into cryovials (Nunc, Thermo Fisher Scientific). Afterwards, the cells were slowly cooled down in a "Mr. Frosty" freezing container (Nalgene, Thermo Fisher Scientific) to -80°C at 1°C per minute. The next day cells were transferred into liquid nitrogen for long term storage.

Frozen cells were thawed quickly in a 37°C water bath and transferred into pre-warmed medium. Then, cells were centrifuged and resuspended in the appropriate medium.

2.2 Cells

2.2.1 Monocyte-derived DCs

This study was approved by the joint University College London/University College London Hospitals National Health Service Trust Human Research Ethics Committee and written informed consent was obtained from all participants. 120 ml of venous blood was sampled in heparinised tubes. Peripheral blood mononuclear cells (PBMCs) were obtained by density-gradient centrifugation (800 x g, 20 min) through Lymphoprep (Axis-Shield). After repeated washing with Hank's buffered saline solution (HBSS) (Gibco), monocytes were isolated through magnetic positive selection using CD14 MACS MicroBeads (Miltenyi Biotec) according to manufacturer's instructions. After purification the isolated cells were ≥ 95% $CD14^+$ cells. Cultures were established in complete RPMI-1640 supplemented with 100 ng/ml recombinant human GM-CSF and 50 ng/ml rhIL-4 (both gifts from Schering-Plough Research Institute, Kenilworth, NJ). Cells were incubated at $5 \times 10^5 - 1 \times 10^6$ cells per ml. After a 4 day incubation period these cells can be used as immature DCs (iDCs). DC cultures were assessed by flow cytometry to show a typical morphology in the forward scatter/side scatter (FSC/SSC) and expression of DC-SIGN. The immature phenotype was confirmed by no CD83 and low CD86 expression. The $CD14^-$ population of PBMCs was cryopreserved and stored in liquid nitrogen.

2.2.2 Monocyte-derived LCs

$CD14^+$ monocytes were obtained the same way as for the generation of human monocyte-derived DCs. Cells were cultured in complete RMPI-1640 supplemented with 100 ng/ml rhGM-CSF, 50 ng/ml rhIL-4 and 10 ng/ml rhTGF-β (Peprotech). On day 4 of culture half of the medium was replaced, containing fresh cytokines. 7 days after the

isolation of monocytes these cells express E-cadherin and Langerin and can be used as immature LCs. The $CD14^-$ population of PBMCs was cryopreserved in FBS containing 10% (v/v) DMSO and stored in liquid nitrogen.

2.2.3 T cells

2.2.3.1 Isolation of total T cells

Cryopreserved $CD14^-$ PBMCs cells (see 2.2.1 and 2.2.2) were thawed quickly at 37°C and equilibrated in pre-warmed medium (RPMI). Trypan blue (Sigma) negative (live) cells were counted and 50 µl CD19 and 50 µl HLA-DR (both supernatants from T cell hybridomas) per 3×10^6 cells were added. After 30 min of incubation on ice the cells were washed once with ice cold HBSS and 10 µl Pan IgG anti-mouse Dynabeads (Invitrogen) per 3×10^6 cells were added. Cells were placed in a rotating mixer for 45 min at 4°C and subsequently placed into a magnet holder (Invitrogen). The supernatant was removed carefully and added into a second tube placed in a magnet holder to remove any remaining cells bound to magnetic beads. Again, the supernatant was removed, washed once with HBSS and the T cells were counted.

2.2.3.2 Isolation of total $CD4^+$ cells

Untouched $CD4^+$ T cells were prepared from frozen $CD14^-$ PBMC using the $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec). Stimulated DCs were harvested and purified via density-gradient centrifugation (800 x g, 20 min) through Lymphoprep. Afterwards cells were seeded at 0, 10, 100, and 1000 DCs/well. 10^5 allogeneic $CD4^+$ T cells were added to the DCs. Cells were cultured in complete RPMI medium.

2.2.4 EpiDerm

Epiderm cultures were obtained from MatTek. Skin samples were taken out of the agarose and placed in a 6-well plate with 2 ml of medium (serum-free DMEM, MatTek) to equilibrate at 37°C over night.

2.2.5 HaCaT cells

The human KC cell line HaCaT, which had derived from spontaneously transformed KCs, was used as an *in vitro* KC model. HaCaT cells were a gif t from A. Reske and cultured in complete DMEM medium (Boukamp et al., 1988). HaCaT cells were grown in tissue culture flasks to 80-90% confluence. Every two or three days cells were split 1:4 or 1:8 respectively. For this, medium was aspirated and cells were washed once with phosphate buffered saline (PBS) (without Ca^{2+} and Mg^{2+}, Lonza, BioWhittaker). In order to remove cells 0.25% Trypsin/EDTA (Invitrogen) was added and cells were incubated at 37°C and 5% CO_2 for 5 min. The reaction was stopped by adding complete DMEM.

2.3 Chemicals

All chemicals were purchased from Sigma (Sigma-Aldrich), except for DMSO (Acros Organics). The classification of the chemicals as sensitisers or irritants according to the LLNA is summarised in Table 2-1 (dos Santos et al.).

Abbrevation	Chemical	Classification
DNFB	1-Fluoro-2,4-dinitrobenzene	Extreme sensitiser
DNCB	1-Chloro-2,4-dinitrobenzene	Extreme sensitiser
DNBS	2,4-Dinitrobenzene-1-sulfonic acid	Strong sensitiser
TNBS	2,4,6-Trinitrobenzene-1-sulfonic acid	Strong sensitiser
DCNB	1,2-Dichloro-4-nitrobenzene	Weak sensitiser
BA	Benzoic acid	Irritant
DMSO	Dimethylsulfoxide	Irritant
SDS	Sodium dodecyl sulfate	Irritant

Table 2-1: Classification of sensitisers and irritants according to the LLNA

2.4 Treatment of HaCaT cells

Cells of the human keratinocyte cell line HaCaT (Boukamp et al., 1988) were trypsinised and $1x10^7$ cells were labelled with different chemicals in 3.3 mL PBS for 1 hour. After the labelling the chemicals were removed by repeated washing (3 x) with PBS and subsequently HaCaT cells were cultured in complete RPMI medium. For coculture experiments with DCs, HaCaTs were seeded at 1.25×10^6 cells/ml; for the production of supernatants HaCaTs were seeded twice as densely. Collected supernatants were filtered and frozen at -80°C. The cytotoxic effect of the chemicals on the HaCaT cells was assessed by flow cytometry, using propidium iodide (PI) as a measure of cell death after 24 hours of culture.

Analysis of DNFB-treated HaCaT cells by flow cytometry showed that DNFB had bound to the HaCaT cells (Figure 2-1).

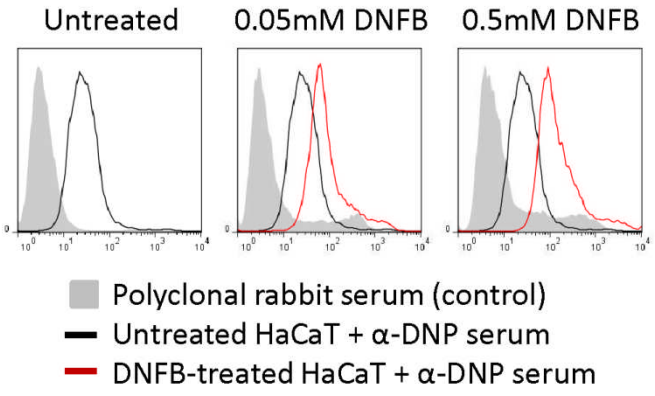


Figure 2-1: DNFB binds to HaCaT cells

HaCaT cells that had been treated with different concentrations of DNFB for 1 hour and subsequently cultured for 24 hours were stained with polyclonal anti-DNP rabbit serum or control polyclonal rabbit serum. FITC-labelled polyclonal $F(ab)_2$ fragments, specific for anti-rabbit Ig, were used as a secondary layer. Unspecific staining of untreated HaCaT cells with the DNP serum was observed, probably due the lack of an isotype control. However, DNFB-treated HaCaT cells showed a dose-dependent increase in the MFI of the DNP-specific staining.

2.5 Treatment of EpiDerm cells

Samples were placed into 2 ml of PBS and the chemicals added to the top of the sample. After 1 hour of incubation at 37°C the samples were washed 5 times with PBS before being placed back into 2 ml of fresh medium and cultured at 37°C for 20 hours. The supernatant was then collected and an MTT assay (see 2.10) performed.

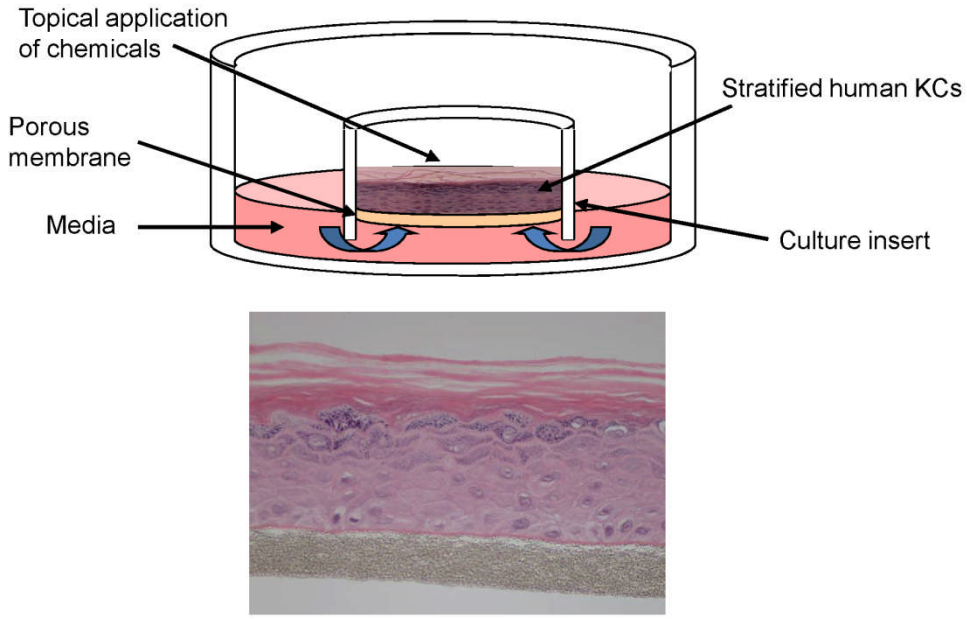


Figure 2-2: Model of an EpiDerm™ culture (modified from Vicki Summerfield)

2.6 Activation of DC and LC

Day 4 immature DCs (iDCs) were stimulated for 20 hours with IL-1α (Peprotech) or LPS (10-100 ng/ml; *Escherichia coli* O111:B4, InvivoGen). Day 7 immature LCs (iLCs) were stimulated with PolyI:C (50 µg/ml, InvivoGen). Alternatively iDCs or iLCs were cocultured with chemically treated HaCaT cells in a ratio of 1:50 for 20 hours. For the activation of iDCs with the supernatants from HaCaT cells the supernatants were diluted 1:2 with fresh medium and then cultured for 20 hours.

For the IL-1α neutralisation experiments, the thawed HaCaT cell supernatants were treated with α-IL-1α (R&D systems) or control antibody (goat IgG) for 30 min at 37°C before the DCs were added in fresh RPMI leading to 1:2 dilution of the HaCaT supernatant. Cells were cultured for 20 hours.

2.7 Uptake of HaCaT cells by DCs

To determine the internalisation of HaCaT cell fragments by DCs, HaCaT cells were labelled with 3' tetra-methyl-indocarbocyanine perchlorate (DiI, Sigma Aldrich) for 30 min at 37°C, 5% CO_2. The final DiI concentration was 5 µM. Then, the HaCaT cells were exposed to the different chemicals for 1 hour (as described in 2.4) and subsequently cells were washed in PBS. DiI labelled and chemical-exposed HaCaT cells were cocultured with DCs for 24 hours. Afterwards, cells were collected and stained for CD13 (as described in 2.9) to distinguish DCs from HaCaT cells. Flow cytometry was used to determine the percentage of HaCaT cells taken up by DCs. To determine non-specific DiI transfer into DCs, parallel cocultures were set up at 4°C for 24 hours.

2.8 Coculture of DCs with allogeneic T cells

iDC were primed for 24 h with different stimuli (see section 2.6), harvested and purified via density-gradient centrifugation (800 x g, 20 min) using Lymphoprep. Afterwards DCs were seeded into round-bottomed 96-well microtiter plates at 0, 10, 100, and 1000 DCs/well. 10^5 allogeneic T cells were added to the DC and cultured for 3 days to determine T cell proliferation or for 5 days to measure cytokine production.

2.9 Flow cytometry

2.9.1 Antibodies

Specificity	Fluorochrome	Isotype	Clone	Manufacturer
CD1a	FITC	mIgG1 κ	HI149	BD Bioscience
CD13	APC	mIgG1 κ	WM15	BD Bioscience
CD14	PE	mIgG2a κ	M5E2	BD Bioscience
CD19	PE	mIgG1 κ	HIB19	BD Bioscience
CD3	FITC	mIgG2a κ	HIT3a	Miltenyi Biotech
CD54 (ICAM-1)	PE	mIgG1 κ	HA58	BD Bioscience
CD80	PE	mIgG1 κ	L307.4	BD Bioscience
CD83	FITC	mIgG1 κ	HB15e	BD Bioscience
CD86	PE	mIgG1 κ	2331 (FUN-1)	BD Bioscience
CD207 (Langerin)	FITC			Gift
CD209 (DC-SIGN)	APC	mIgG2b κ	DCN46	BD Bioscience
CD273 (PD-L2)	APC	mIgG1 κ	MIH18	BD Bioscience
CD274 (PD-L1)	APC	mIgG1 κ	MIH1	BD Bioscienc
IL-1RI	PE	goat	Polyclonal	R&D Systems
HLA-DR	FITC	mIgG2a κ	L243 (G46-6)	BD Bioscience
Annexin V	APC	mIgG2a κ	VAA-33	eBioscience
Anti-DNP serum	non	Rabbit serum	Polyclonal	
Anti-rabbit	FITC	Swine $F(ab)_2$	Polyclonal	Dako

Table 2-2: Antibodies for flow cytometry

2.9.2 Cell surface staining

To test the phenotypic maturation, stimulated DCs and the appropriate controls were incubated in blocking buffer (HBSS containing 10% (v/v) goat serum and 0.05% (v/v) sodium azide) for 1 hour at 4°C, to block non-specific binding. Subsequently, cells were stained for 30 min at 4°C in 50 µl blocking buffer containing the diluted antibodies (Table 2-2) or matched isotype controls. Afterwards, cells were washed twice in HBSS and resuspended in 100 µl FACS buffer (HBSS containing 1% (w/v) BSA and 0.05% (v/v) sodium azide). Prior to analyses of the samples propidium iodide (PI) (eBioscience) was added according to manufacturer´s instructions. Live DCs were distinguished from HaCaT cells by gating on the DC-SIGN positive cells and by PI exclusion. Similarly LCs were identified by $CD13^+$-staining.

HaCaT cells were stained for AnnexinV-APC and PI according to manufacturer´s instructions (eBioscience) in order to quantify cells undergoing apoptosis/necrosis. Samples were analysed using a FACSCalibur (BD Bioscience) flow cytometer and FlowJo software (Tree Star, Inc) was used to analyse the data.

2.10 MTT assay

5 mg of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was suspended in PBS and afterwards diluted 1:5 in culture medium. 2 ml of the diluted MTT was added into 6-well plates and the skin samples were cultured for 3 hours at 37°C. Then, the samples were transferred into 2 ml of isopropanol (Sigma Aldrich) and 1 ml of isopropanol was added onto the top of the sample. The plates were placed on a shaker for 1 hour at room temperature. To obtain all the cells, the cells were removed from the insert and left in the isopropanol. 200 µl of each sample was read in triplicate on a Spectrophotometer at 570nm.

2.11 T cell proliferation

T cell proliferation induced by coculture of allogeneic T cells with stimulus-primed-DC (see section 2.6) was measured by adding 1 µCi of [^{3}H] thymidine (ICN Biomedical) on day 2 of coculture. Cells were harvested after 18 hours and T cell proliferation was assessed by liquid scintillation counting (Microbeta Systems). Results were expressed as counts per minute (cpm).

2.12 Enzyme-linked Immunosorbent Assay (ELISA)

2.12.1 Antibodies

All antibodies were purchased from eBioscience.

Specificity	Type	Clone	Concentration
IFNγ	capture	NIB42	2µg/ml
	detection	4S.B3	1µg/ml
IL-13	capture	PVM13-1	2µg/ml
	detection	13-7138	1µg/ml
IL-17A	capture	eBio64CAP17	1µg/ml
	detection	eBio64DEC17	1µg/ml
IL-1α	capture	CRM8	2µg/ml
	detection	CRM6	1µg/ml

Table 2-3: Antibodies for ELISA

2.12.2 Protocol

ELISA plates (Nunc) were coated with 50 µl/well capture antibody (Table 2-3) overnight at 4°C. Plates were washed 3 times with washing buffer (0.05% Tween-20 in PBS). To block any free residues, wells were incubated with 2% (w/v) bovine serum albumin (BSA, Sigma) for 1 hour at room temperature (RT). After washing 3 times in washing buffer, the plates were incubated with duplicates of 50 µl sample per well for 2 hours at RT. Standard was prepared by diluting recombinant human proteins (rh) in RPMI (rhIFNγ, Peprotech). Two-fold serial dilution was performed to produce a standard curve. Duplicates of the standard were incubated for 2 hours at RT (50 µl/well), as for the samples. The plates were washed 3 times and incubated with 50 µl/well detection antibody (Table 2-3) in washing buffer for 1 hour at RT. After 3 more washes in washing buffer the wells were incubated with 50 µl of 1:250 diluted avidin-horseradish peroxidise (avidin-HRP) (eBioscience) for 30 min at RT. After 5 more washing steps HRP presence was detected by adding 100 µl tetramethylbenzidine (TMB) (eBioscience) substrate for about 10 min in the dark. To stop the reaction, 50 µl

1N HCl were added. Colorimetric reactions were quantified immediately on the MRX Revelation plate reader at 450 nm, using the Revelation v4.21 software (Dynex Technology) for analysis.

2.13 Multiplex analysis of cytokines

Stored supernatants from DC-HaCaT cocultures or HaCaT cell cultures were used to quantify the production of 25 different human cytokines, chemokines and growth factors using human Biosource multiplex bead immunoassay kits according to manufacturer's instructions (Invitrogen). The 25-Plex assay allows the simultaneous measurement of the following proteins: IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-13, IL-15, IL-17, TNF-α, IFN-α, IFN-γ, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, Eotaxin, RANTES, and MCP-1. The multiplex analysis was performed using Luminex 200IS^{TM} platform (Luminex). Concentrations were determined using the LuminexIS software 2.3 (Luminex).

2.14 SDS polyacrylamide-gel ectrophoresis (SDS-PAGE)

2.14.1 Sample preparation

HaCaT cells were labelled with different chemicals and cultured in serum-free AIM-V medium for 20 hours. **Supernatants** were collected and concentrated using a centriprep column (Millipore, 3000 x g, 30 min). 4x reducing sample buffer was added (0.0625M Tris-HCl (pH6.8) 10% Glycerol, 2% SDS, 5% 2-mercaptoethanol, bromophenol blue). **Cell pellets** were washed in PBS (Gibco, Invitrogen) before being lysed in reducing sample buffer. Samples were sonicated for 20-30 seconds on ice at 40 Hz using an ultrasonic processor (Jencons Scientific) and then heated to 98°C for 5 min in order to shear the genomic DNA.

2.14.2 Electrophoresis

Cell lysates and concentrated supernatants were loaded onto an Invitrogen gel (for 10 ml of a **10% acrylamide running gel:** 4 ml H_2O, 3.3 ml 30% acrylamide, 2.5 ml 1.5 M Tris (pH 8.8), 0.1 ml 10% SDS, 0.1 ml 10% APS, 4 µl TEMED), for **stacking gel**: 5 ml H_2O, 1 ml 30% acrylamide, 2.5 ml 1.0 M Tris (pH 6.8), 0.1 ml 10% SDS, 0.1 ml 10% APS, 10 µl TEMED) and run in **running buffer** (14.4 g Glycine, 3 g Tris-base, 1 g SDS in 1 litre of deionised water) at 150 V in a Xcell SureLock Moni-Cell electrophoresis system (Invitrogen) until the dye reached the bottom of the gel. The prestained protein ladder PageRuler (Fermentas, York, UK) was run with the samples to determine the size of the proteins.

2.15 Western-Blotting

2.15.1 Protein transfer

Following SDS-PAGE the stacking gel was removed and the running gel was equilibrated in transfer buffer (14.4 g Glycine, 3 g Tris-base in 1 litre of deionised water) for 10 min. Amershan Hybond ECL nitrocellulose membranes (GE Healthcare, Little Chalfont, UK) were briefly soaked in transfer buffer before the protein transfer was set up in a Xcell SureLock Mini-Cell electrophoresis system using the blot module (Invitrogen) at 100 V for 1 hour.

2.15.2 Blocking

The membranes were briefly washed in washing buffer (TBS/Tween) and afterwards incubated in TBS/Tween containing 5% (w/v) skimmed milk powder (Tesco) for 1 hour at RT or overnight at 4°C to block non-specific binding sites.

2.15.3 Blotting

The membranes were washed briefly in washing buffer before adding the primary antibody diluted in TBS/Tween, containing 1% (w/v) skimmed milk powder (Tesco) for 1 hour at RT or overnight at 4°C. After three 5 minute washes in washing buffer the membranes were incubated with a HRP-conjugated secondary antibody (diluted in TBS/Tween, containing 1% (w/v) skimmed milk powder (Tesco)) for 1 hour at RT. Afterwards, the membranes were washed 3 times in washing buffer, followed by 3 washes in TBS (without Tween). Primary and secondary antibodies used in this study are documented in Table 2-4.

Specificity	conjugate	Host	Clone	Dilution	Manufacturer
Anti-human HMGB-1	None	Rabbit	Polyclonal	1:200	eBioscience
Anti-human GAPDH	None	Rabbit	GAPDH-71.1	1:10000	Sigma Aldrich
Anti-human β-actin	None	Mouse	AC-15	1:10000	Abcam
Anti-mouse HRP	HRP	Goat	Polyclonal	1:2000	DAKO
Anti-rabbit HRP	HRP	swine	Polyclonal	1:2000	DAKO

Table 2-4: Antibodies for Western Immunoblotting

2.15.4 Development

The membrane was covered with ECL Western blotting detection reagent (GE Healthcare) according to manufacturer's instructions. ECL was drained and the membrane was wrapped in cling film before chemiluminescence was measured by autoradiography using ECL Hyperfilm (GE Healthcare), which was developed in a Developer Xograph imaging system CompactX4. Primary and secondary antibodies were stripped off by incubating the membrane in stripping buffer at 60°C for 45 min. Afterwards, the membranes were intensively washed in washing buffer and blocked before being re-probed for different proteins.

2.16 Transcriptional profiling using microarrays

$1x10^6$ immature DCs were seeded, and either left unstimulated or stimulated with 10 ng/ml IL-1α for 4 hours. The supernatant was collected and the cells were lysed in RLT buffer (Qiagen, Crawley, UK) and stored at -80°C until the RNA was purified using a RNeasy Mini Kit (Qiagen, Crawley, UK) and following manufacturer's instructions. The RNA was eluted in 30 µL RNase-free water (Qiagen, Crawley, UK) and contaminating DNA was removed using the TURBO DNA-*free* kit from Ambion (Austin, TX, USA).

A RNA 600 Nano assay (Agilent, Santa Clara, CA, USA) and a bioanalyzer (Agilent 2100 Bioanalyzer, Santa Clara, CA, USA) were used to electrophoretically determine the amount and integrity of the RNA following manufacturer's instructions. Only samples with a RIN higher than 7 were used for subsequent experiments.

500 ng of RNA were converted into double-stranded cDNA, which was then amplified into cRNA using T7 RNA polymerase, incorporating Cyanine 5 (Cy5)-/Cyanine 3 (Cy3)-labelled CTP fluorescent dyes (Agilent's Quick Amp Labeling Kit, PerkinElemer Life and Analytical Sciences, Boston, MA, USA). Qiagen's RNeasy mini spin columns were used for purification of the labelled cRNA samples, which were eluted in 30 µL of nuclease-free H_2O (Qiagen). The purity and yield of the cRNA as well as the labelling intensity were measured using a NanoDrop ND-1000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA yield had to be higher than 825 ng and the labelling intensity more than 8 pmol Cy3/Cy5 per µg cRNA in order to proceed. The samples were fragmented and hybridised onto Agilent 4 x 44K whole human genome cDNA microarrays, according to manufacturer's instructions. The array slides were scanned at a resolution of 5 µm using an Agilent dual-laser microarray scanner G2565BA, and the Agilent Feature Extraction software

(v9.5.1) was used to acquire the signal intensities of the different probes from the array image. The function "AAProcess" in the R package agilp was used to subject the raw data to a $\log_2$ transformation, LOESS normalisation and removal of duplicates (Chain et al., 2010). Principal component analysis was performed using the R package "PCA prcomp.R". Normalised data from stimulated DCs were compared to a set of 13 unstimulated DC data sets, collected in our laboratory by different researchers over the past four years, using MultiExperiment Viewer v4.4.1. Genes which changed significantly ($p<0.05$), and by more than 2-fold compared to the unstimulated samples, were selected for further analysis. The open-access bioinformatics tool Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to perform functional annotation cluster analysis (Dennis et al., 2003). Enriched transcription factor binding sites were identified using the bioinformatics tool oPOSSUM (Ho Sui et al., 2005).

2.17 Statistical analysis

Repeated measure (RM) 2 way ANOVA or paired/unpaired student's t-test were used where appropriate using the GraphPad Prism Software. Data were log transformed before statistical analysis if the standard deviation was not invariant.

2.17.1 Regression analysis

HaCaT cells were treated with titrations of different chemicals. The concentrations of the chemicals (x-values) were $\log_{10}$ transformed in GraphPad Prism and plotted against the percentage of PI positive cells. A non-linear regression line (log(agonist) vs response – variable slope) using a four-parameter logistic equation was fitted and the hill slope (which denotes the steepness of the dose-response curve), logEC50 (which is the logarithm of chemical concentration that leads to a response half way between the

minimal and maximal response) and R^2 (which denotes the goodness of fit) were determined using GraphPad Prism.

DCs stimulated with HaCaT cell supernatant were analysed for CD83 and CD86 expression, which were plotted against the percentage of PI positive HaCaT cells or $\log_{10}$ transformed chemical concentrations. A linear regression line was fitted and the slope of the correlation line, the goodness of fit (R^2) and the chemical concentration or amount of cell death resulting in a 20% increase in CD83/CD86 expression were determined using the GraphPad Prism Software.

3 *IN VITRO* MODEL FOR SKIN SENSITISATION

3.1 Introduction

Our skin is exposed to many different chemicals, which we encounter through nature, cosmetic products, and fabrics or at work. Despite the very effective protection that is provided via the skin, certain chemicals, which lead to modification of skin proteins by covalently binding to it, are the causative agents of CHS reactions. It is therefore of great importance that cosmetic products are tested for their potential to induce allergic reactions after skin contact. However, concerns about the use of animals for cosmetic product testing led to a European Directive aiming to replace the LLNA by alternative *in vitro* methods in 2013. One approach to distinguish between sensitisers and irritants is to dissect DC responses after exposure to different chemicals. DCs are at the forefront of an immune response and play a pivotal role in the initiation and maintenance of adaptive immune responses. It is thought that DCs respond to environmental and antigenic signals in the skin and might be able to distinguish between sensitiser- and irritant induced changes. This should lead to differential activation profiles (e.g. cytokine production, surface marker expression) and translate into specific T cell responses. However, the irritancy properties of many sensitisers and the high concentrations necessary to obtain an effect leave only a narrow window of concentration in which the effect of sensitisers and irritants can be assessed. Furthermore, it has been suggested that some cytotoxicity or external stimulus like TLR ligation is needed in order to see DC stimulation (Basketter and Maxwell, 2007). In order to provide a more controlled system we sought to develop a two cell system in which the KC is exposed to the chemical first before coculture with the DC.

3.2 Objectives

- Study the direct effect of DNFB on DCs.
- Set up a KC-DC coculture model.
- Investigate the hypothesis that sensitisers can act as PAMPs and activate DCs, by:
    - Testing if sensitiser-treated HaCaTs lead to DC maturation.
    - Testing if irritant-treated HaCaTs lead to DC maturation.
    - Testing if the supernatant from sensitiser- or irritant-treated HaCaTs is sufficient to induce DC maturation.

3.3 Direct effect of DNFB onto DCs

Activation of LCs in the epidermis and dDCs in the dermis plays a crucial role in the initiation of cutaneous immune responses. Monocytes were obtained from PBMCs of healthy volunteers and cultured in GM-CSF and IL-4 for 4 days to obtain mo-DCs or in GM-CSF, IL-4 and TGF-β for 7 days in order to obtain mo-LCs. Mo-DC showed a typical DC morphology and did not express the monocyte marker CD14 or the T- and B cell markers CD3 and CD19, respectively (Figure 3-1A). However, CD1a, MHC class II and the DC marker DC-SIGN were highly expressed (Figure 3-1A). Mo-LCs did not express DC-SIGN, but stained positive for the LC markers E-cadherin and Langerin (CD207) (Figure 3-1B). Both DCs and LCs upregulated CD83 and CD86 after stimulation with TLR ligands, such as LPS and PolyI:C (Figure 3-1A+B). To test if the sensitiser DNFB could stimulate DCs directly, mo-DCs were incubated with increasing concentrations of DNFB overnight (20 hours) and activation (CD83/CD86 expression) as well as cell necrosis (propidium iodide (PI) staining) was measured. Only the highest

concentration of DNFB augmented the expression of the DC activation markers. However this concentration also induced a considerable amount of cell death (Figure 3-2). Necrotic cells have been reported to stimulate DCs (Newton et al., 2003; Sauter et al., 2000). It can therefore not be excluded that the dying cells activated the DCs rather than the DNFB itself. As mentioned previously, we therefore wanted to establish a two cell model to study the effect of sensitisers and irritants on DCs.

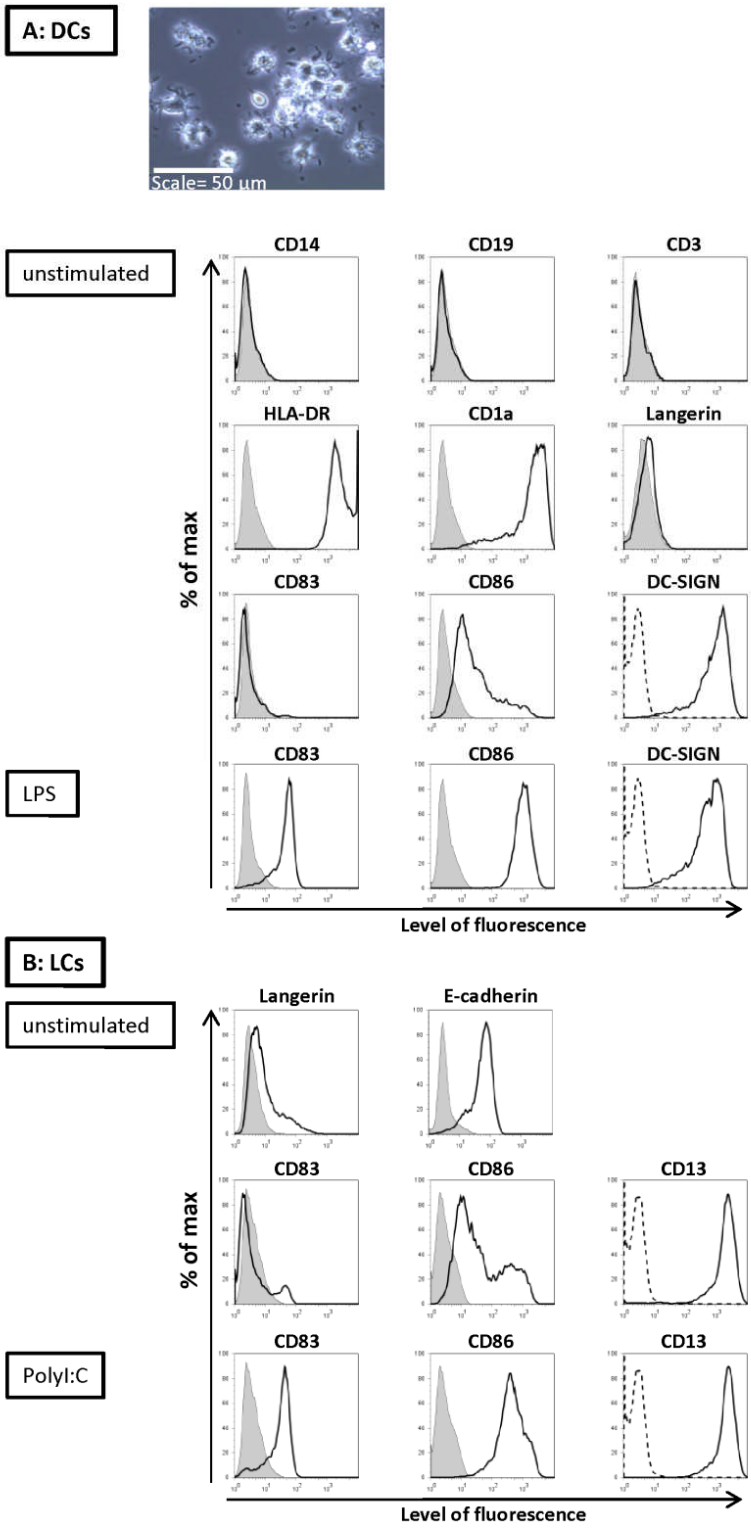


Figure 3-1: Phenotypic analysis of monocyte-derived dendritic cells and Langerhans cells

A: Light microscopy image of day 4 monocyte-derived DCs. Unstimulated and LPS stimulated monocyte derived DCs were stained with the indicated antibodies and expression measured by flow cytometry. **B:** Unstimulated and PolyI:C stimulated LCs were stained with the indicated antibodies and expression measured by flow cytometry. Shaded graphs: isotype control, solid line: indicated marker on DCs/LCs, dashed line: indicated marker on HaCaT cells. One of two representative experiments is shown.

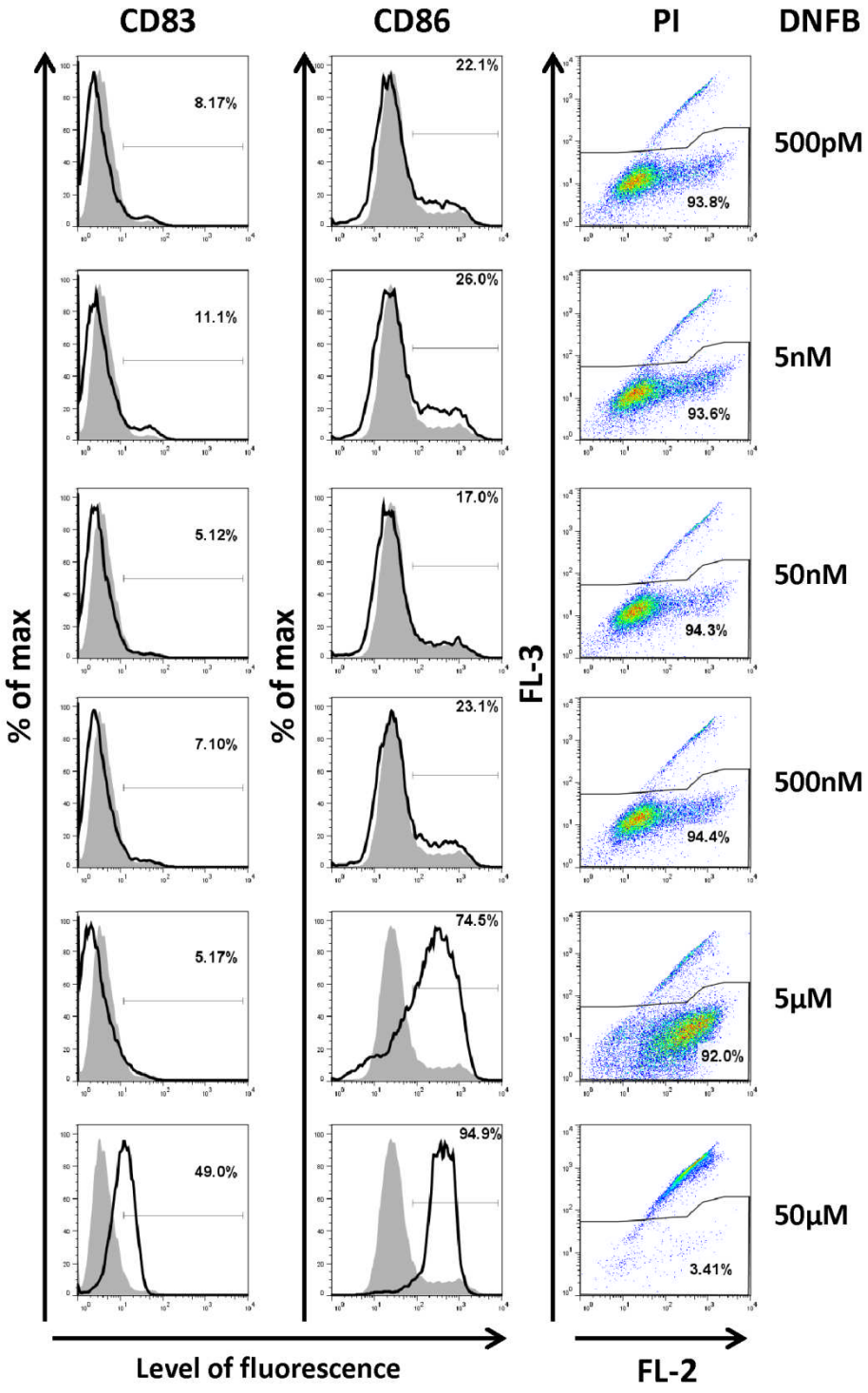


Figure 3-2: High concentrations of DNFB induce DC maturation, but are cytotoxic.

Increasing concentrations of DNFB were directly added to cultured iDCs. CD83 and CD86 expression and PI uptake were assessed by flow cytometry 24 hours later. Shaded graphs show unstimulated DCs. Black lines represent DCs exposed to indicated concentrations of DNFB. Live cells were determined as being double negative in FL-2 and FL-3.

3.4 Coculture of DC/LC with DNFB treated HaCaTs leads to activation of DC/LC

We used the well-known human cell line HaCaT as a model for KCs. The cells were exposed to increasing concentrations of sensitisers or irritants for 1 hour. The chemicals were washed off and cells were cultured for 24 hours before the cytotoxic effect was assessed by PI staining. DNFB, DNCB, TNBS and DNBS are sensitisers and BA, DMSO and SDS are classified as irritants. DCNB is either regarded as a very weak sensitiser or an irritant (Table 2-1). The % of PI positive cells was plotted against sensitiser concentration (Figure 3-3), and regression lines fitted as described in chapter 2.17.1. The EC50 values were calculated from the regression lines, for each chemical and are shown in Table 3-1.

	DNFB	DNCB	TNBS	DNBS	DCNB	SDS	BA	DMSO
LogEC50	-1.12	0.03	0.74	1.73	0.52	-0.51	n.d.	3.51
Hill slope	3.19	2.38	4.77	3.65	3.99	12.28	n.d.	20.21
EC50 (mM)	0.08	1.08	5.49	53.12	3.29	0.31	n.d.	3220.00
R^2	0.95	0.95	0.66	0.79	0.86	0.57	n.d.	0.84

Table 3-1: Chemical-induced cytotoxicity of HaCaT cells.

The titration curves of chemical-induced cytotoxicity of HaCaT cells as shown in Figure 3-3 were analysed in GraphPad Prism using a non-linear regression (four-parameter logistic equation). The hill slope, logEC50, EC50 and R^2 value for each dose-response curve are summarised in this table.

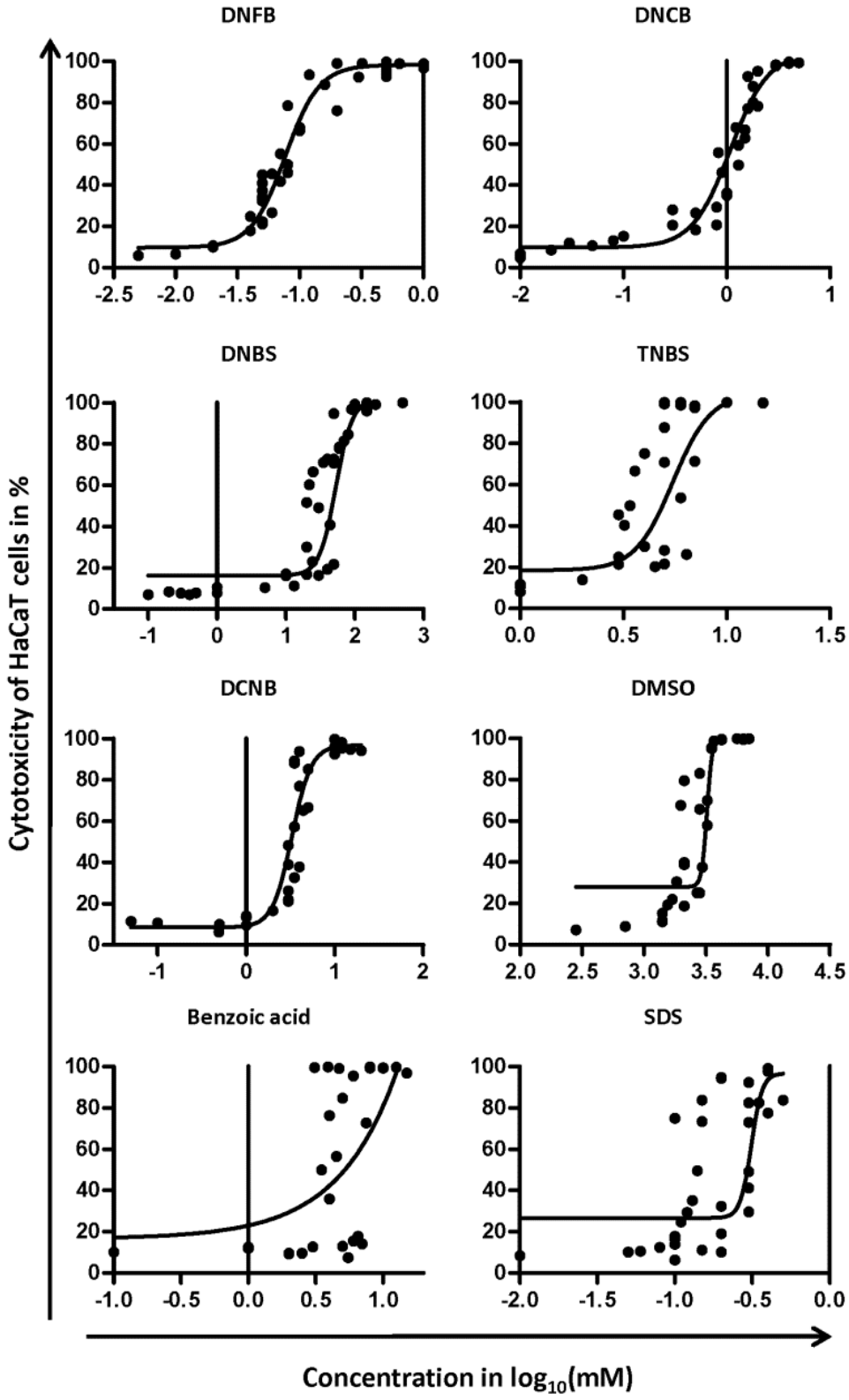


Figure 3-3: Chemical-induced cytotoxicity of HaCaT cells

HaCaT cells were incubated with different concentrations of the different chemicals for 1 hour. Chemicals were washed off and cells were cultured at 37°C, 5% CO_2 for 20 hours. Cells were stained with PI and the percentage of PI positive cell was plotted against log_{10} transformed chemical concentrations. Regression lines were drawn in GraphPad Prism using a nonlinear regression (four-parameter logistic equation). Data of several independent experiments are summarised in these graphs.

Costaining with Annexin V and PI after 1 hour or 24 hours of culture revealed that the majority of cells underwent necrosis after DNCB, DNFB and DMSO treatment. A small proportion of cells was apoptotic after 1 hour (AnnexinV positive, PI negative population) but then went into secondary necrosis (PI positive population) (Figure 3-4).

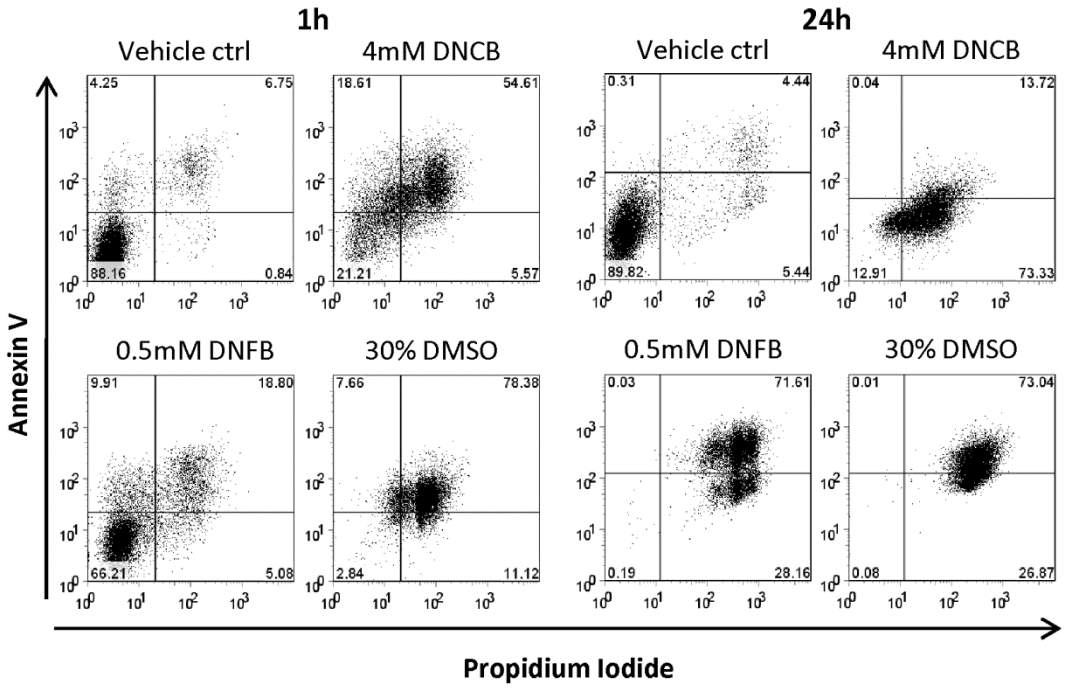


Figure 3-4: Sensitisers and irritants mainly induce necrosis in HaCaT cells

HaCaT cells were treated with different chemicals for 1 hour. Chemicals were washed off and cells were cultured at 37°C, for 1 or 24 hours, before they were stained with Annexin V-APC and PI. Annexin V positive, PI negative cells are undergoing apoptosis. PI positive cells are necrotic. One of three representative experiments is shown.

HaCaT cells were treated with 0.05mM or 0.5mM DNFB, which resulted in 25-30% and 100% cell death, respectively. After chemical exposure and three washes in PBS the DNFB-labelled HaCaT cells were cocultured with DCs or LCs in a ratio of 1 DC/LC to 10 HaCaTs or 1 DC/LC to 50 HaCaTs for 20 hours. DC-SIGN expression of DCs and CD13 expression of LCs was used to distinguish them from HaCaT cells (Figure 3-5A). Dead cells were excluded by PI staining (Figure 3-5A). Coculture of DNFB-treated HaCaT cells with DCs or LCs resulted in augmented expression of the activation markers CD83 and CD86 compared to cocultures of HaCaT cells treated with the vehicle control and DCs/LCs (Figure 3-5B, Figure 3-6). Culture with HaCaT cells treated with the higher concentration of DNFB resulted in a higher expression of CD83/CD86 on the DCs/LCs compared with the lower concentration of DNFB. Additionally, CD83 and CD86 expression was further augmented when higher numbers of HaCaT cells were added, as can be seen in the 1:50 ratio as opposed to a ratio of 1:10 DC:HaCaT (Figure 3-5B, Figure 3-6). CD86 expression on DCs/LCs cocultured with 0.5mM DNFB-treated HaCaT cells in a ratio of 1:50 was almost as high as for cells stimulated with LPS or PolyI:C as a positive control, showing a robust DC/LC activation (Figure 3-5B, Figure 3-6). The high cell numbers needed to stimulate DC/LC activation suggested that soluble factors are involved.

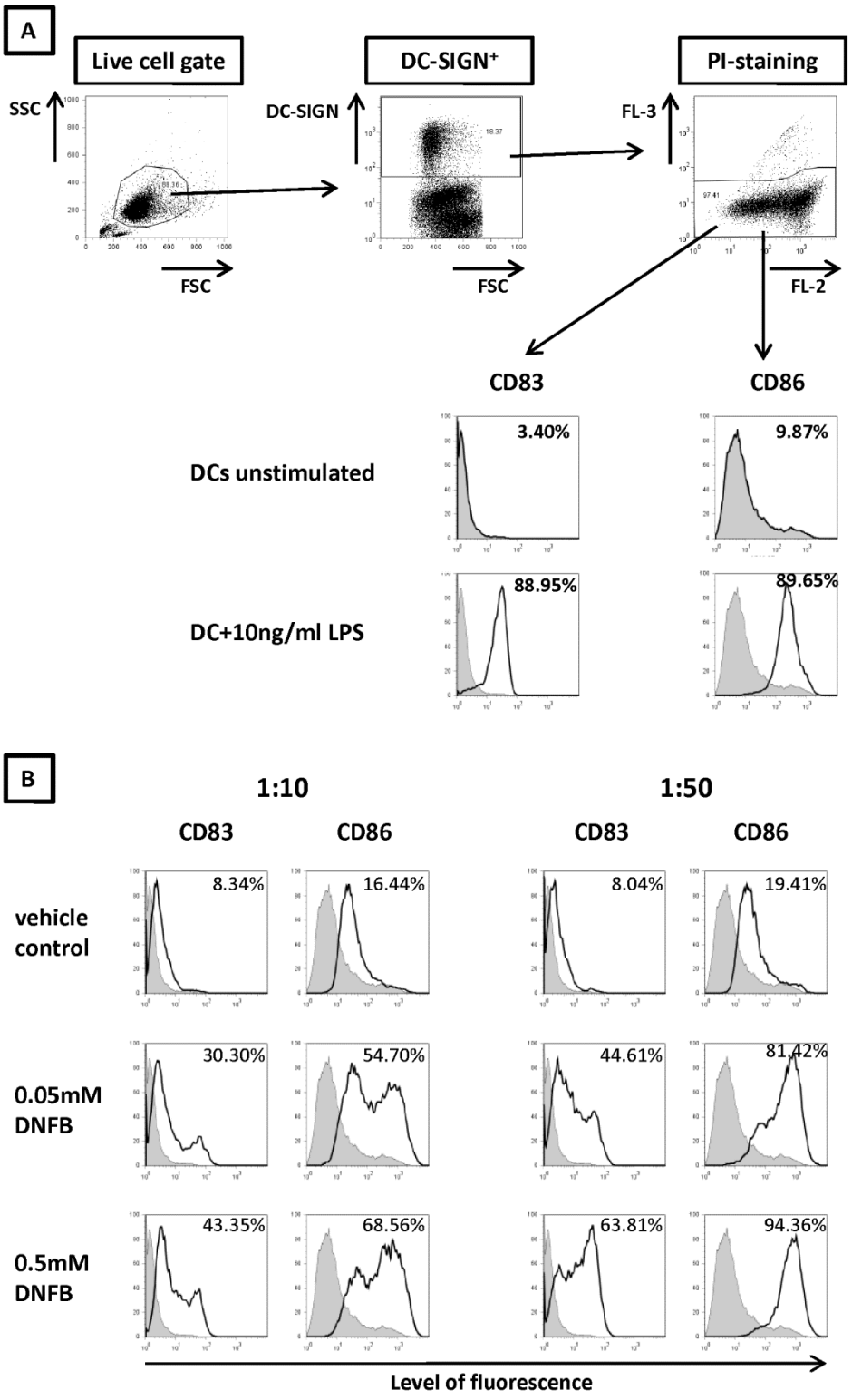


Figure 3-5: DC maturation after coculture with DNFB-treated HaCaT cells

HaCaT cells were labelled with DNFB for 1 hour and then cocultured with monocyte-derived DCs for 20 hours, cells were analysed by flow cytometry. **A:** gating strategy: live cell gate (left panel), DC-SIGN expression distinguishes DCs from HaCaTs (middle panel), live cells are identified as PI-negative cells (right panel), CD83 and CD86 expression of live, DC-SIGN$^+$ cells is analysed, cells stimulated with 100 ng/ml LPS serve as positive control **B:** DCs and DNFB-labelled HaCaT cells were cocultured at a ratio of 1:10 and 1:50. CD83 and CD86 expression of DCs of one representative experiment is shown. Summary of several experiments is shown in Figure 3-6.

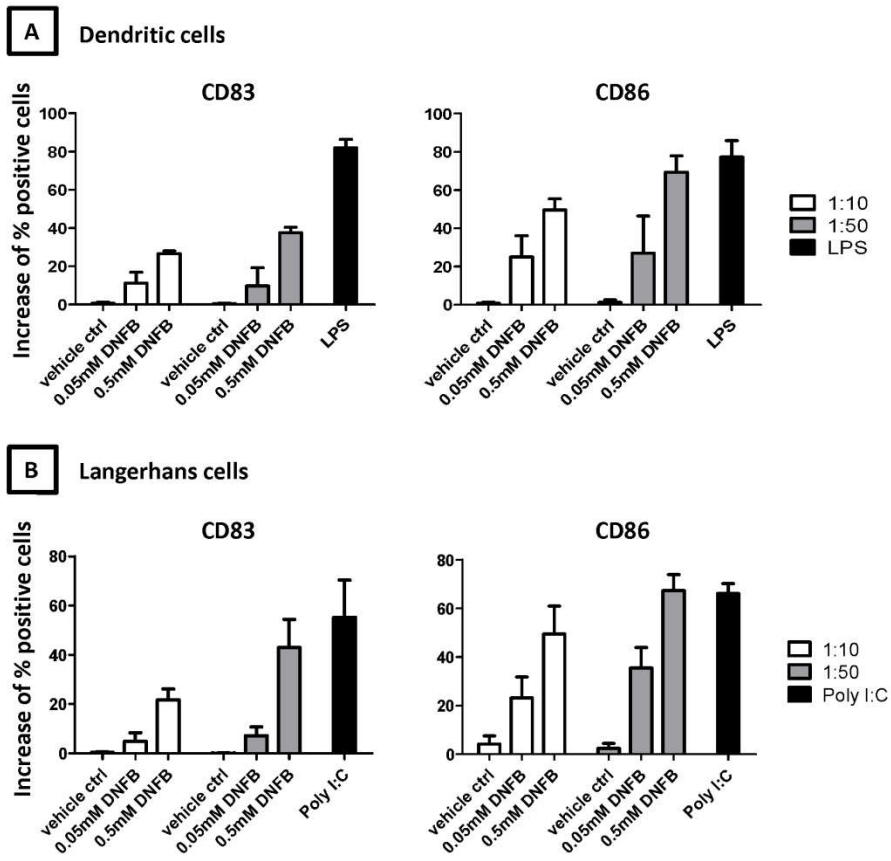


Figure 3-6: DC and LC maturation after coculture with DNFB-treated HaCaT cells

HaCaT cells were labelled with DNFB for 1 hour and then cocultured with DCs (A) or LCs (B) for 20 hours. DCs/LCs were analysed by flow cytometry for CD83 and CD86 expression as shown in Figure 3-5. DCs/LCs were stimulated with 100ng/ml LPS or 50µg/ml PolyI:C as a positive control. **A:** the mean of 4 independent experiments is shown, error bars: SEM **B:** the data range of 2 independent experiments is shown, error bars: SEM

HaCaT cells were labelled with the fluorescent dye DiI before chemical treatment in order to determine whether DCs take up the dying cells. DCs that had been cocultured with chemical-treated HaCaTs at 37°C for 24 hours showed costaining with DiI, indicating that the DCs had taken up the dying HaCaT cells (Figure 3-7). An identical experimental setup was performed at 4°C, in order to determine passive diffusion of dye into the DCs. However, this control easily gives false-positive results when the temperature is only slightly increased, enabling the DCs to phagocytosis. This explains why the background is slightly higher than expected.

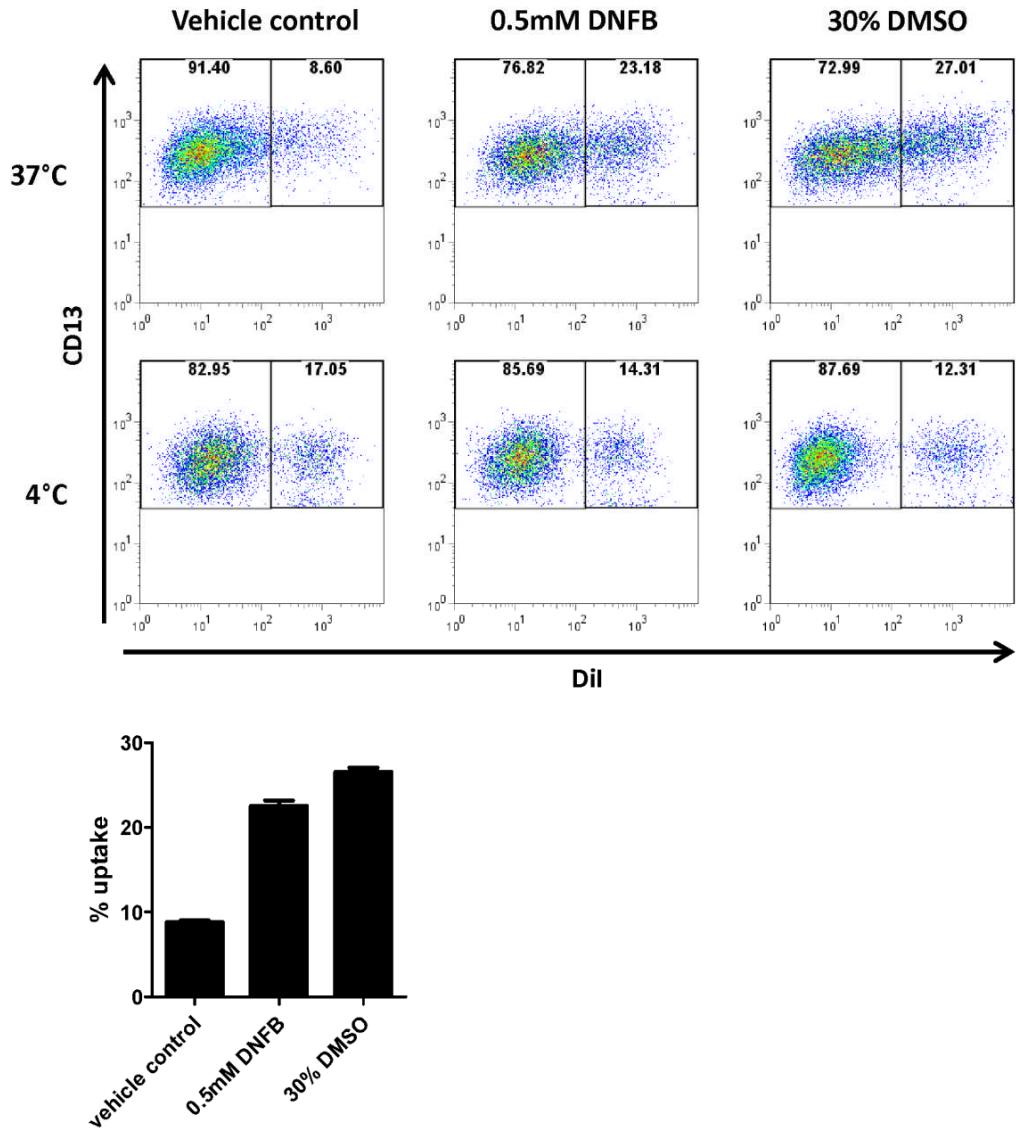


Figure 3-7: Uptake of dying HaCaT cells by DCs.

HaCaT cells were labelled with DiI and afterwards treated with 0.5mM DNFB or 30% DMSO for 1 hour. HaCaT cells were then cocultured with DCs at a ratio of 1:1 for 20 hours at 37°C. An identical control experimental setup was performed at 4°C to determine unspecific uptake of cell fragments. Uptake of DiI^+ HaCaT cell fragments by DCs ($CD13^+$) was determined by flow cytometry. The median and data range of 2 different DC donors is shown.

HaCaT cells treated with high concentrations of DNCB, another sensitiser, and DMSO, an irritant, also led to augmented expression of the DC activation markers CD83 and CD86, similar to DNFB (Figure 3-8A). The concentrations tested initially in these experiments led to 100% HaCaT cell death after 20 hours of culture (Figure 3-3).

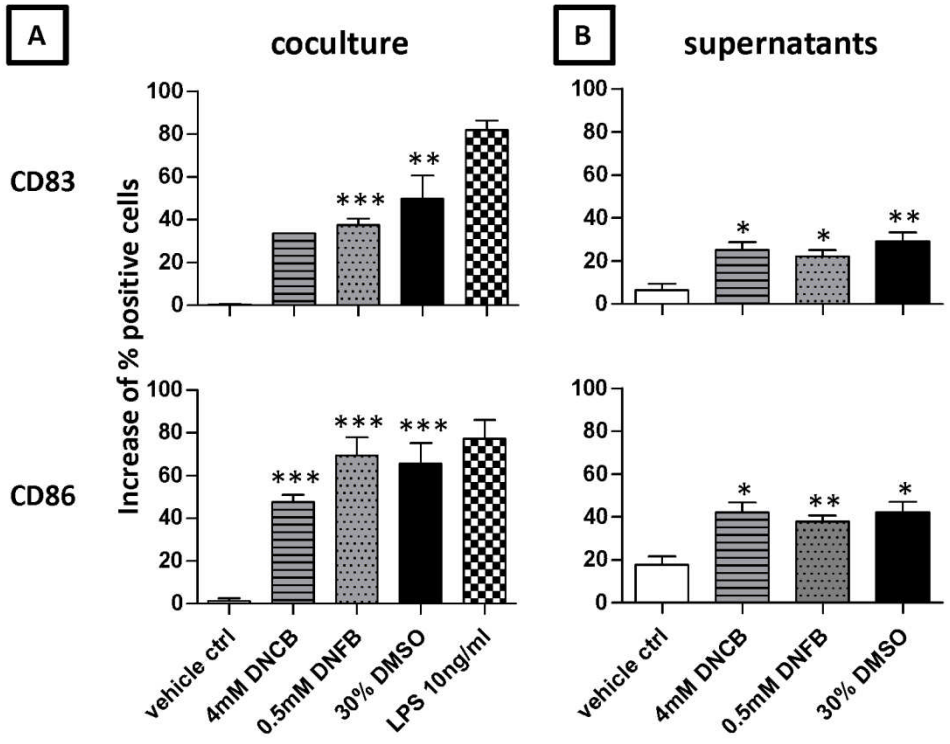


Figure 3-8: Activation of DCs with sensitiser/irritant-treated HaCaTs

HaCaT cells were treated with the different chemicals for 1 hour. The chemicals were washed off and HaCaT cells were either cocultured with iDC at a ratio of 50:1 for 20 hours **(A)** or cultured on their own. Supernatants were collected after 20 hours, which were then used to stimulate DC **(B)**. Increase in CD83 and CD86 expression was assessed by flow cytometry and is shown relative to unstimulated cells. Data of 2-4 experiments are shown as means+SEM **(A)**. Data of ≥11 experiments are shown as means+SEM **(B)** * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (paired student's t-test, compared to vehicle control).

3.5 The supernatant of chemical-treated HaCaT cells is sufficient to stimulate DC activation

In order to gain insight into the activation of DCs by chemical-treated HaCaT cells, supernatant of HaCaTs treated with 0.05mM and 0.5mM DNFB was collected after 3, 6 and 24 hours of culture. The HaCaT supernatant had to be diluted 1:2 before addition to the DCs. Therefore the HaCaT cells were seeded twice as densely compared to the coculture experiments. The supernatant of chemical-treated HaCaT cells was sufficient to induce CD83 and CD86 upregulation (Figure 3-8, Figure 3-9). The later the supernatant was collected the more CD83/CD86 expression was induced, suggesting

that the activating agent might be slowly released from the dying cells (Figure 3-9A). Heat-inactivation of the supernatants at 56°C for 30 min or freeze-thawing (-80°C) did not alter the ability of the supernatant to induce DC maturation, measured by CD83 and CD86 expression (Figure 3-9B). Similar to DNFB, supernatant from DNCB and DMSO-treated HaCaT cells was sufficient to induce DC maturation (Figure 3-8B). However, coculture with chemical-treated HaCaT cells augmented the expression even further, suggesting a role for cell-cell interaction. Again, no difference between the sensitisers and irritants was found (Figure 3-8A+B).

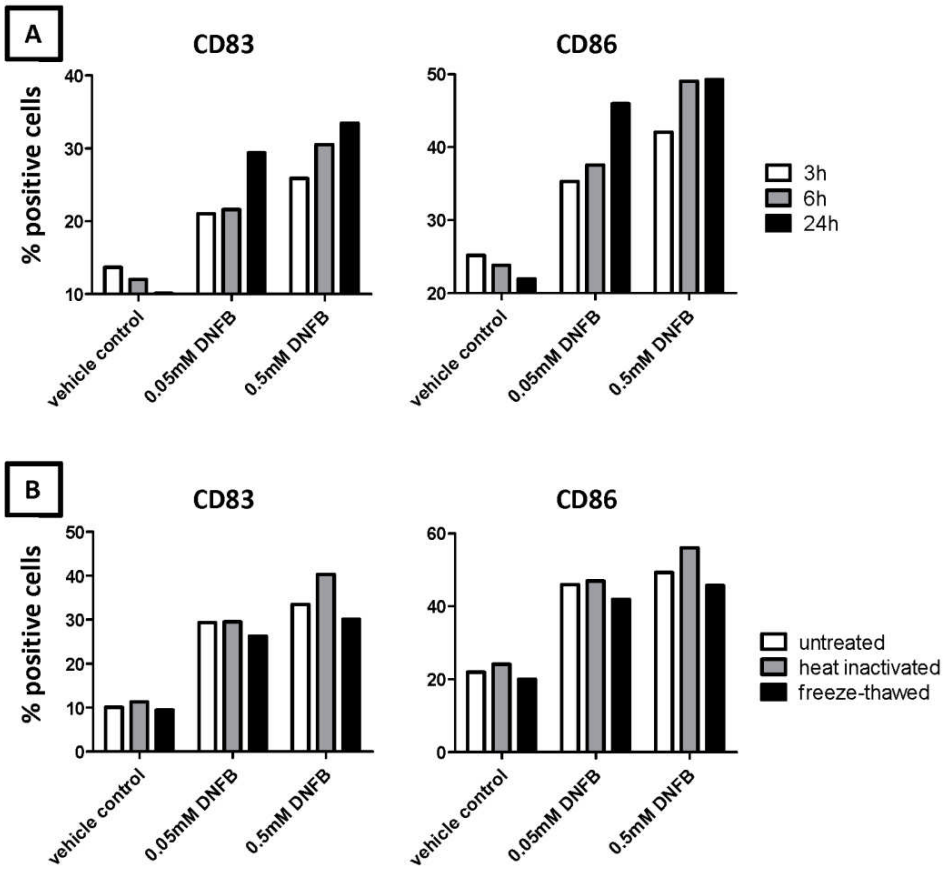
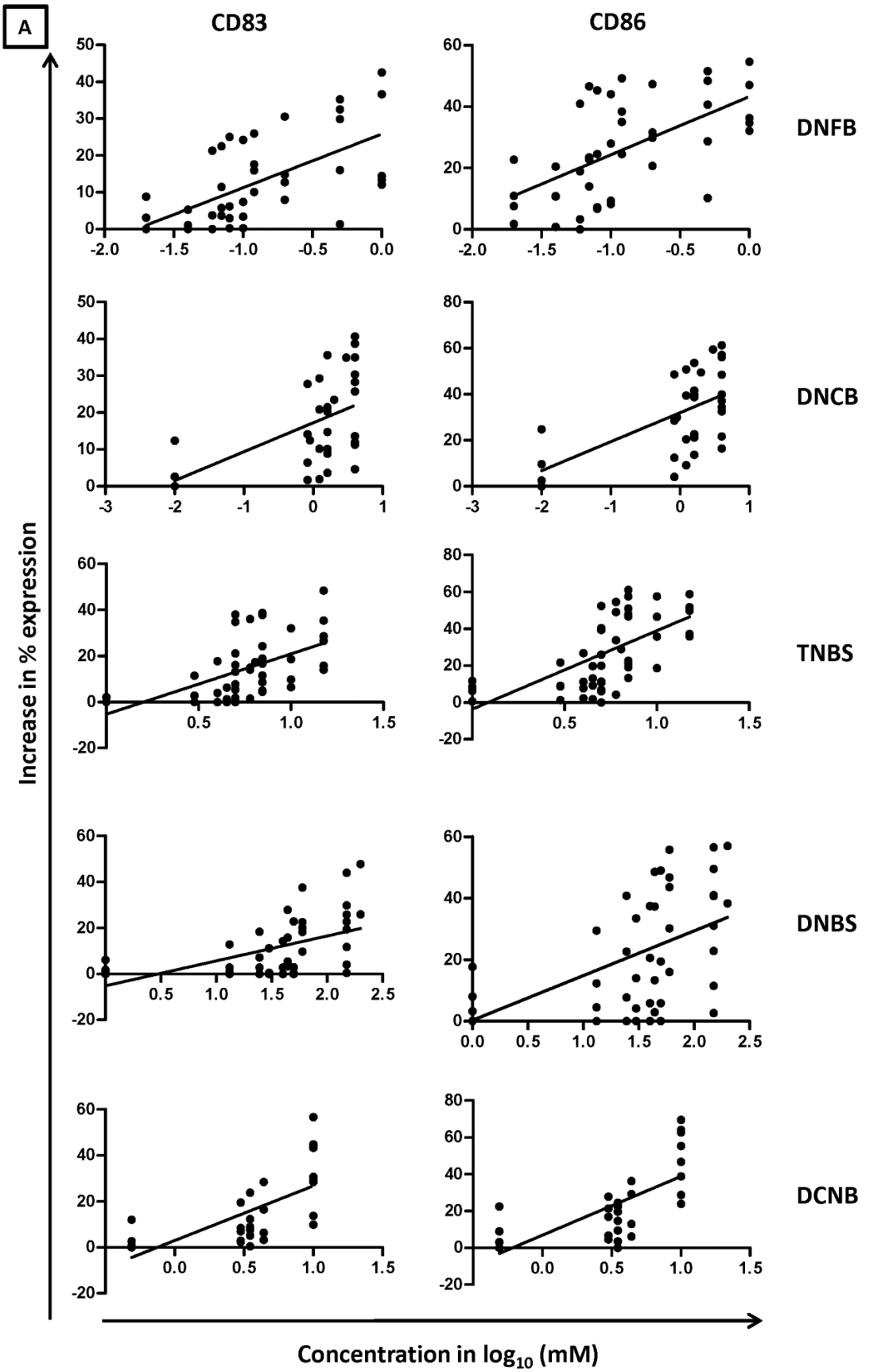


Figure 3-9: Supernatant of DNFB-treated HaCaTs induces DC maturation

HaCaT cells were labelled with DNFB for 1 hour. Chemicals were washed off and HaCaT cells were then cultured at 37°C, 5% CO_2. Supernatants were filtered, diluted 1:2 and added to iDCs. The ratio of DCs to supernatant from HaCaT cells is 1:50. **A:** Supernatants were collected after 3, 6, and 24 hours. DCs were stimulated with the supernatants for 20 hours, thereafter CD83 and CD86 expression was measured. **B:** Supernatants were collected after 24 hours and either heat-inactivated at 56°C, 30 min, or freeze (-80°C) – thawed. DCs were stimulated with the supernatants for 20 hours, thereafter CD83 and CD86 expression was measured. Bars show percentage of positive cells compared to unstimulated DCs. One of two representative experiments is shown **(A+B)**.

In order to determine whether quantitative, rather than qualitative differences might be observed between the effects of sensitisers and irritants, supernatants from HaCaT cells treated with a wide range of concentrations of both sensitisers (DNCB, DNFB, TNBS, DNBS, DCNB) and irritants (SDS, BA, DMSO) were produced. These supernatants were then tested on DCs as described above. Significant variation both in toxicity and in DC stimulation was observed in samples from different experiments. However, overall the amount of DC maturation (as measured by CD83 and CD86 upregulation) was positively correlated to the concentrations and induced toxicity for all chemicals tested except SDS (Figure 3-10). The amount of cytotoxicity induced in the HaCaTs due to the chemical treatment correlated better with the increase in CD83/CD86 expression than the concentrations of the chemicals used (Figure 3-10A versus B, Table 3-2, Table 3-3), reflecting the experimental variation seen in Figure 3-3. Neither the correlation constant, nor the level of cytotoxicity required to reach an enhancement of 20% was significantly different between sensitisers and irritants (Table 3-2, Table 3-3).


A
CD83
CD86
DNFB
DNCB
TNBS
DNBS
DCNB
Increase in % expression
Concentration in log10 (mM)

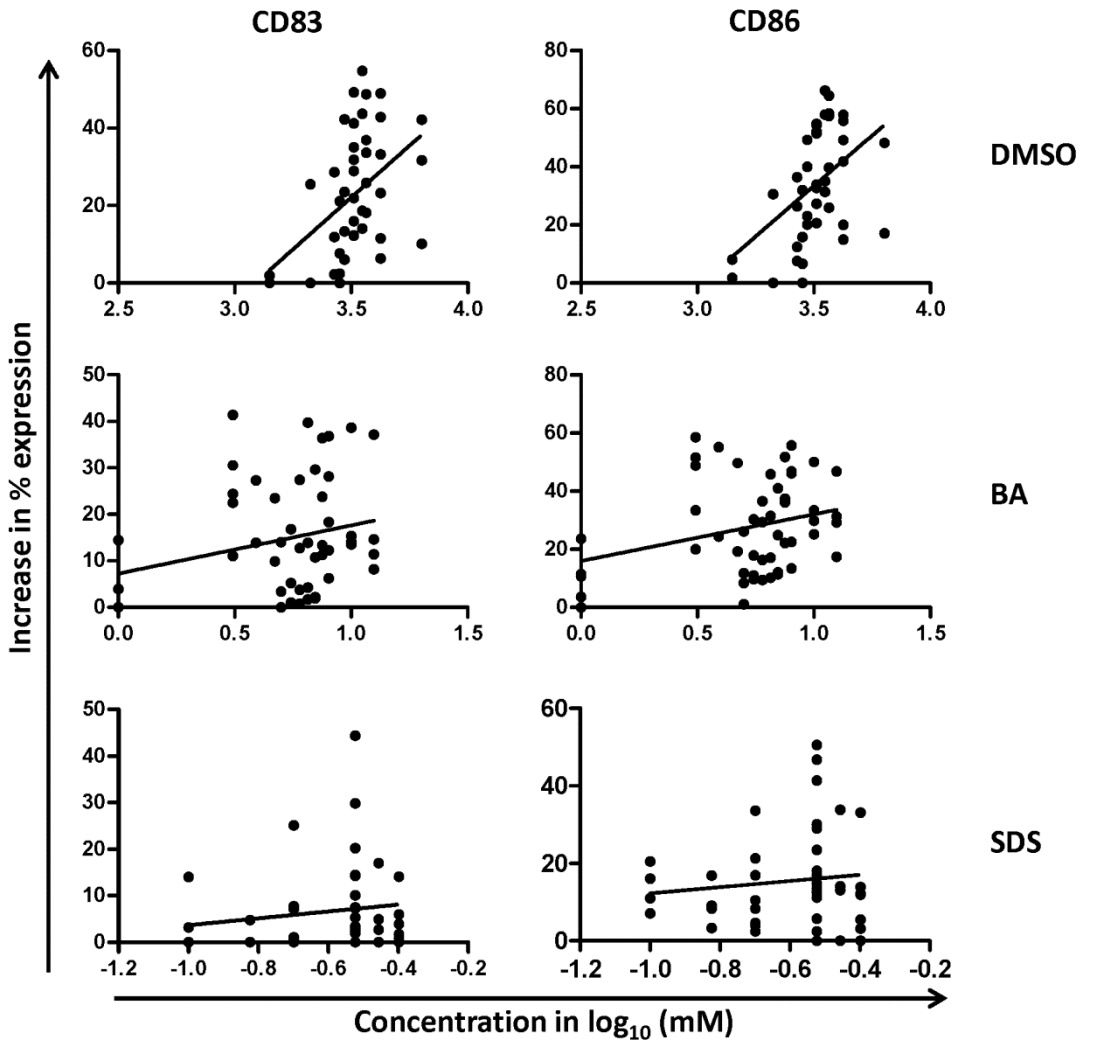
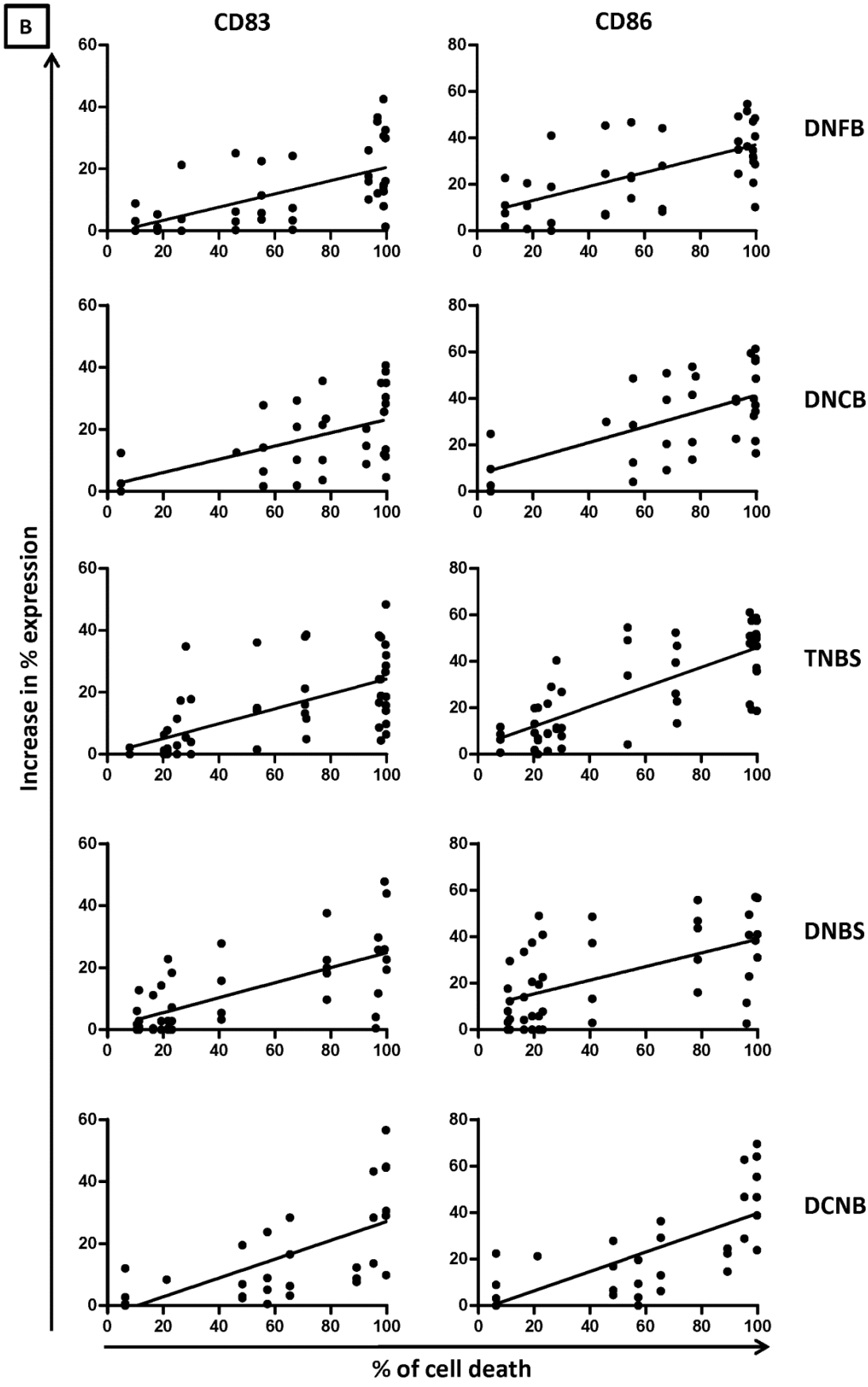


Figure legend can be found on page 110.


B
CD83
CD86
DNFB
DNCB
TNBS
DNBS
DCNB
Increase in % expression
% of cell death

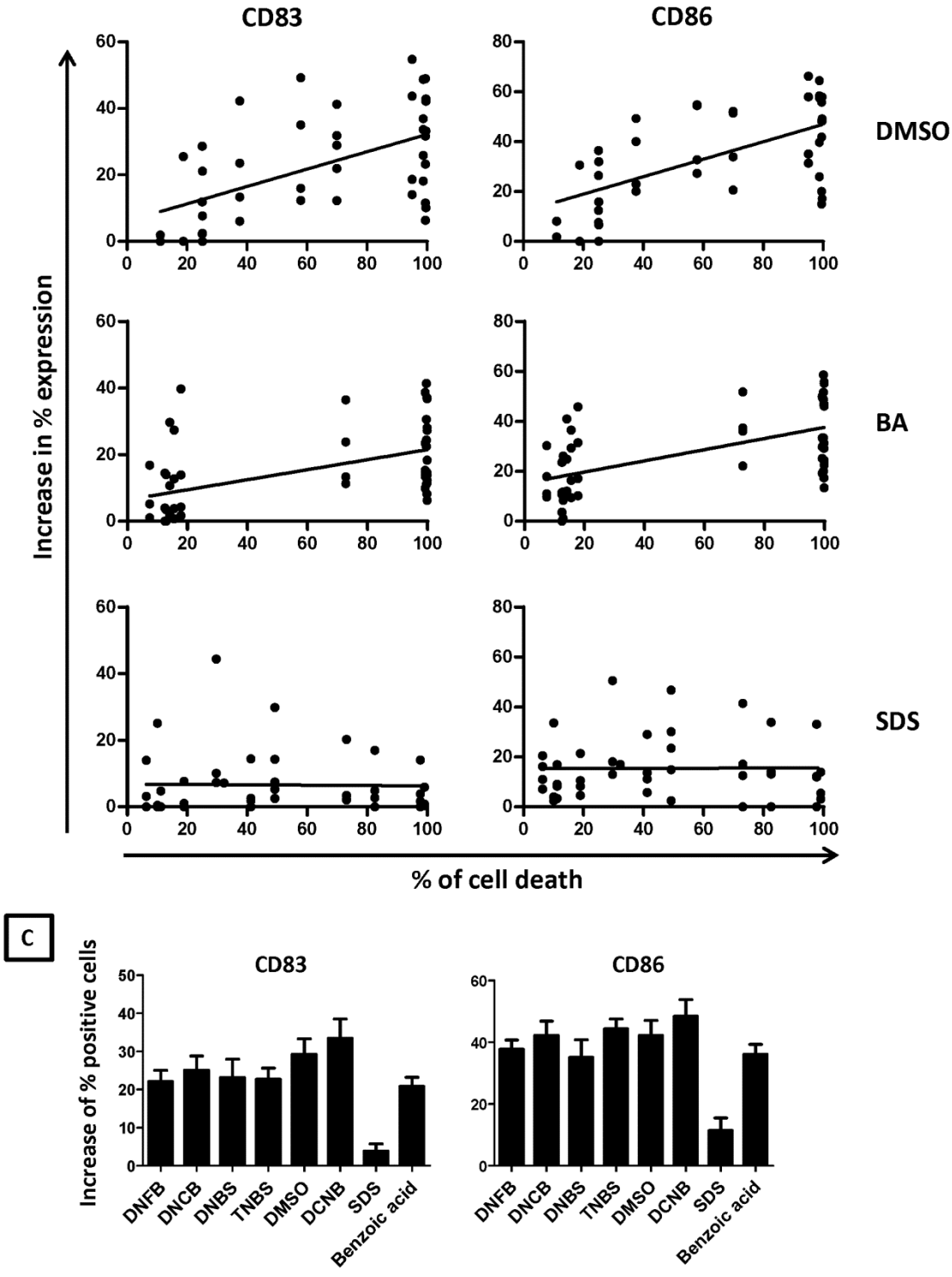


Figure 3-10: DC activation by supernatants of chemically treated HaCaT cells

HaCaT cells were labelled with different chemicals for 1 hour. Chemicals were washed off and HaCaT cells were then cultured at 37°C, 5% CO_2. Supernatants were filtered, and stored at -80°C. They were diluted 1:2 before addition to iDCs. The ratio of DCs to supernatant from HaCaT cells was 1:50. CD83 and CD86 expression was measured after 20 hours. Collected data of several independent experiments is shown. **A:** Increase in CD83/CD86 expression plotted against chemical concentrations. **B:** Increase in CD83/CD86 expression plotted against % cell death induced in HaCaT cells. **C:** same as B, but only data leading to >97% cell death in the HaCaTs are shown. Linear regression lines were drawn using GraphPad Prism.

chemical	Surface marker	Slope	R square	Chemical concentration (mM) resulting in 20% increase of CD83/CD86 expression
DNFB	CD83	14.54 ± 2.96	0.38	0.41
	CD86	19.01 ± 4.10	0.35	0.06
DNCB	CD83	7.84 ± 2.39	0.26	2.20
	CD86	12.57 ± 3.34	0.32	0.11
TNBS	CD83	26.18 ± 5.58	0.30	9.21
	CD86	42.57 ± 7.58	0.38	3.57
DNBS	CD83	10.80 ± 2.89	0.25	200.00
	CD86	14.55 ± 4.34	0.21	22.78
DCNB	CD83	23.65 ± 5.61	0.40	5.31
	CD86	31.87 ± 7.01	0.43	2.575
DMSO	CD83	53.46 ± 16.9	0.20	2898.31
	CD86	69.49 ± 19.8	0.23	2020.11
BA	CD83	10.37 ± 5.92	0.06	n.d.
	CD86	16.05 ± 7.61	0.08	1.79
SDS	0.08	7.37 ± 7.91	0.02	n.d.
	0.95	7.95 ± 10.65	0.01	n.d.

Table 3-2: Correlation between chemical concentration and CD83/CD86 expression on DCs

A linear regression analysis of the data in Figure 3-10A was performed using GraphPad Prism.

chemical	Surface marker	Slope	R square	% HaCaT cell death resulting in 20% increase of CD83/CD86 expression
DNFB	CD83	0.21 ± 0.04	0.37	97.79
	CD86	0.30 ± 0.06	0.40	43.15
DNCB	CD83	0.21 ± 0.06	0.28	85.45
	CD86	0.34 ± 0.09	0.34	37.04
TNBS	CD83	0.24 ± 0.04	0.39	82.37
	CD86	0.42 ± 0.05	0.58	39.25
DNBS	CD83	0.24 ± 0.04	0.45	80.21
	CD86	0.29 ± 0.07	0.31	35.52
DCNB	CD83	0.30 ± 0.07	0.39	76.36
	CD86	0.42 ± 0.09	0.44	53.02
DMSO	CD83	0.26 ± 0.06	0.29	53.58
	CD86	0.35 ± 0.07	0.37	23.50
BA	CD83	0.15 ± 0.04	0.27	89.64
	CD86	0.22 ± 0.04	0.35	21.22
SDS	0.08	-0.01 ± 0.04	0.00	n.d.
	0.95	0.001 ± 0.06	0.00	n.d.

Table 3-3: Correlation between HaCaT cell death and CD83/CD86 expression on DCs

A linear regression analysis of the data in Figure 3-10B was performed using GraphPad Prism.

3.6 Discussion

3.6.1 Irritancy

In vitro alternatives for testing the allergic potential of chemicals are urgently needed in order to replace the need for animal test data to inform consumer safety risk assessments. Many studies have been undertaken to investigate the direct effect of sensitisers on DCs (Aiba et al., 2003; Aiba et al., 2000; Aiba et al., 1997; Arrighi et al., 2001; De Smedt et al., 2005; Hulette et al., 2005; Manome et al., 1999; Pichowski et al., 2000; Tuschl et al., 2000). However, many sensitisers show irritancy effects and cause cytotoxicity of the target cell, making the dose setting and the study of the direct effect of sensitisers on DCs challenging.

Therefore, we wanted to establish a two cell model to control for the cytotoxicity of DCs. KCs are the cells of choice as any skin penetrating agent will first come into contact and possibly react with this cell type. LCs in the epidermis and possibly DCs in the dermis are therefore likely to encounter sensitisers in the context of KCs. Furthermore, they are known to release an array of cytokines and chemokines and will therefore shape cutaneous immune responses (Albanesi et al., 2001; Ansel et al., 1990; Lebre et al., 2003). So far, only one other study has attempted to study the effect of sensitisers on DC in the presence of KCs (Schreiner et al., 2007). However, the DC were cultured in KC-medium altering their phenotype to “DC-like” cells (Schreiner et al., 2007, 2008). Furthermore, the chemicals were added directly to the cocultures and could therefore have toxic effects not only on the KCs, but also on the DCs.

We chose the commonly used and widely studied HaCaT cells as a model for KCs, which has also been used to study the sensitising potency of low molecular weight chemicals (Van Och et al., 2005). Increasing concentrations of both sensitisers and

irritants resulted in augmented cytotoxicity of KCs. The titration curves were very steep for all compounds tested, meaning the threshold for cytotoxicity is very sharp. Many sensitisers have irritant effects, which can augment their immunogenic properties, by lowering the response threshold (Allenby and Basketter, 1989, 1993). A trend has been reported for chemicals with more irritant properties to also be more potent sensitisers (Basketter et al., 2007). However, there are a number of compounds that do not follow this correlation, such as the detergents SDS or sodium lauryl sulphate (SLS), which do not show any sensitising potency, but are highly irritating.

3.6.2 Activation of DCs

Cocultures of chemical-treated HaCaT cells with DCs or LCs augmented their expression of the activation markers CD83 and CD86. Dinitrophenols (DNPs) bound irreversibly to HaCaT cells and DCs phagocytosed the moribund HaCaTs. The uptake of parts of the dying cells led to an augmented DC activation compared to supernatants alone. It has been reported that sensitiser-treated KCs shed so called “blebs”, which contained haptenated keratins, namely K5 and K14 (Bauer et al., 2011). Mice treated which such haptens (bromobimanes) produced anti-keratin antibodies, suggesting that hapten binding results in the formation of immunogenic neo-antigens (Simonsson et al., 2011), which could maybe play a role in DC activation. However, we did not investigate this mechanism further.

The supernatant of chemical-treated HaCaTs was sufficient to induce DC maturation, suggesting the release of soluble factors which contribute to DC activation. Heat-inactivation of the supernatants of chemically-killed KCs did not prevent DC activation, suggesting that the activating agents are quite heat-stable. Heat-inactivation at 56°C for 30 min is usually used to inactivate components of the complement system in blood serum before it is used for culturing of cells. Heat-treatment under these

conditions is thought to destroy the tertiary and quartiary structure of many proteins. The nature of the activating agent in the supernatants was studied further in the following chapter.

We were unable to identify any differences between strong or weak sensitisers or irritants, despite exposing HaCaT cells to a whole range of concentrations of different chemicals before adding their supernatants to the DCs. Even at low chemical concentrations which induced little cell death we could not observe any sensitiser-specific activation of DCs, through recognition with a PRR for example. We think it therefore unlikely, that sensitisers are recognised as PAMPs by APCs. The only exception was SDS, which is often used as a negative control when comparing sensitisers to irritants. However, the unique detergent properties of SDS might somehow inactivate the activating agents and should be used with caution when general statements about irritants are being made.

However, our findings are in line with the danger hypothesis, suggesting that innate cells respond to danger, rather than distinguishing only specific classes of pathogens (Ibrahim et al., 1995; Matzinger, 1994). Therefore, we suggest that during the early stages of ACD hapten induced danger signals activate LCs and DCs in the skin to migrate to the lymph nodes and present antigen sampled in the skin to T cells. The idea that APCs in the skin respond to local trauma is supported by the observation that LCs become activated and leave the skin during irritant contact dermatitis (ICD) (Cumberbatch et al., 1993; Willis et al., 1990). ICD is induced by sufficient irritant stimulus and is hard to distinguish from ACD in terms of its morphology. It is characterised by skin barrier disruption, cellular epidermal changes and the release of mainly proinflammatory cytokines (Smith et al., 2002). Opposed to ACD, the response to irritant signals is completely unspecific and not driven by antigen-specific T cells.

3.7 Conclusion

- Development of a novel two cell *in vitro* model to study the interaction between DCs and KCs in the presence of different chemicals
- Coculture of chemically-killed KCs with DCs induces DC maturation
- The supernatant of chemically-killed KC is sufficient to induce DC maturation
- DC maturation correlates with the amount of cell death that is induced
- Sensitisers and irritants behave similarly (except for SDS). We therefore, conclude that sensitisers (tested in this study) do not act as PAMPs.

4 MECHANISMS FOR ACTIVATION OF DCS BY CHEMICAL-TREATED HACAT CELL SUPERNATANTS

4.1 Introduction

In the previous chapter we established a KC-DC coculture model. Coculture of sensitiser- or irritant-killed HaCaT cells with DCs leads to DC activation. Furthermore, the supernatant of the chemical-treated HaCaT cells was sufficient to induce DC maturation. Despite thorough study, we did not identify any differences in DC activation (CD83/CD86 expression) induced by sensitisers or irritants. It therefore seems very unlikely that sensitisers act as PAMPs and specifically activate DCs. Chemical-induced cytotoxicity of KCs might, however, induce the release of danger signals (or DAMPs) from dying cells. DCs have been reported to respond to danger signals.

4.2 Objectives

- Analyse the supernatants of chemical-treated HaCaT cells.
- Analyse the supernatants of DCs stimulated with chemical-treated HaCaT cells or their supernatant.
- Investigate the role of IL-1α in activating DCs in this *in vitro* model through blocking experiments.
- Study the effect of DCs stimulated with chemical-treated HaCaT cells on T cell function.

4.3 Results

4.3.1 Danger signals are released by dying KCs

In the previous chapter we reported the development of a new DC-HaCaT coculture model, whereby chemical-treated HaCaT cells led to DC activation. Furthermore, we showed that the supernatant of these chemical-treated HaCaT cells was sufficient to induce DC maturation. In order to dissect the mechanism by which the DCs are activated further, the supernatants of chemical-treated HaCaT cells were analysed. We used luminex analysis, in which 25 different cytokines and chemokines can be measured simultaneously. Live HaCaT cells that had been treated with the vehicle control showed constitutive production of low levels of CCL5, and high levels of CCL2, IL-6 and IL-8 (Figure 4-1A). Upon chemical treatment, which resulted in 100% cell death after 20 hours of culture, the production of these cytokines and chemokines was prevented (Figure 4-1A, Figure 3-4). On the contrary, IL-1α, and IL-1RA release was augmented following DNFB, DNCB and DMSO treatment (Figure 4-1A+C), supporting previous reports showing that IL-1α and IL-1RA are stored in KCs and released upon necrotic cell death (Gahring et al., 1984). We were also interested in the chromatin-binding protein HMGB-1, which has been suggested to function as a danger signal and has been implicated in sepsis (Wang et al., 1999b). Supernatants of untreated, DNFB or DNCB-treated HaCaT cells were collected after 24 hours in culture, concentrated to about one tenth of the volume and tested by immunoblotting. Necrotic DNFB and DNCB-treated HaCaT cells released HMGB-1 and GAPDH (as a marker of cytoplasm) (Figure 4-1B). In contrast, HaCaTs treated with the vehicle control did not release HMGB-1, but retained it within the cell.

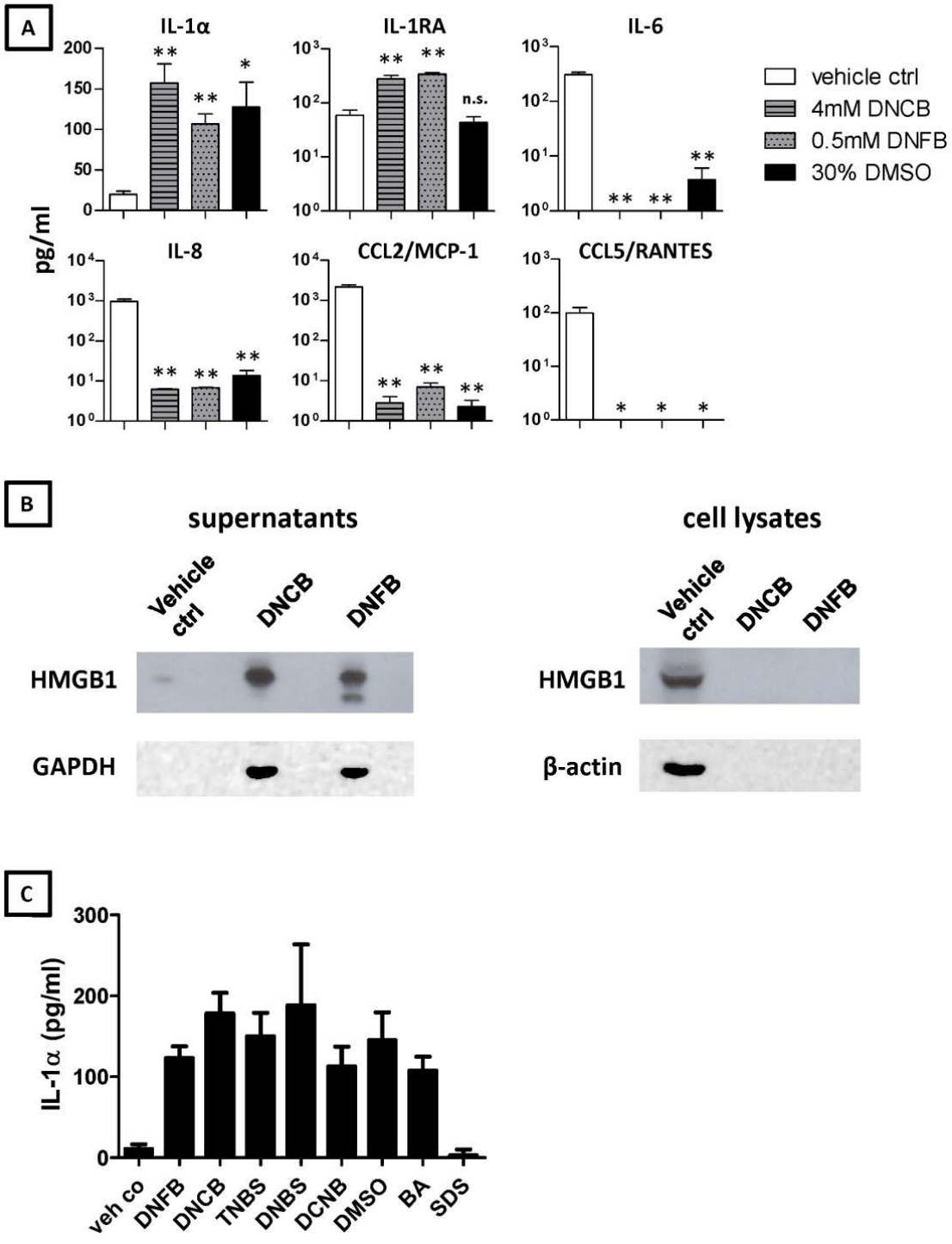


Figure 4-1: Cyokine production of chemical treated HaCaT cells.

HaCaT cells were treated with the different chemicals for 1 hour and then cultured for 24 hours. **A:** Supernatants were analysed by Luminex or ELISA (IL-1α). (n≥4), data are shown as mean+SEM; n.s. not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (paired student's t-test, compared to vehicle ctrl). **B:** HaCaT cells were cultured in serum-free medium for 24 hours. Cell lysates and concentrated supernatant were separated on a SDS-PAGE and plotted for HMGB-1 and GAPDH or β-actin. One of two representative experiments is shown. **C:** As in A, but IL-1α was measured following HaCaT cell treatment with a wider panel of chemicals.

4.3.2 Human skin samples release IL-1α after sensitiser/irritant treatment

In order to confirm the release of IL-1α in a physiologically more relevant model than the immortalised HaCaT cell line, we used Epiderm™ skin models. These are 3D skin-like tissue structures derived from primary human epithelial KCs, which are known as reconstructed human epidermis as they consist of organised basal, spinous, granular, and cornified layers. First, a preliminary experiment was conducted in order to determine the concentrations of the chemicals resulting in 100% cell death, as measured by MTT assay. Then, Epiderm™ skin samples of 3 different donors were treated topically with 4mM DNCB, 2mM DNFB or 100% DMSO for 1 hour. The chemicals were washed off and the skin samples were cultured for another 20 hours. The supernatants were used to measure the release of IL-1α (Figure 4-2A) and a MTT assay (Figure 4-2B) was conducted to determine the amount of cell death. Using this model, we demonstrated that primary KCs release IL-1α when exposed to cytotoxic concentrations of chemicals, although the Epiderm™ model showed high level of interdonor variability.

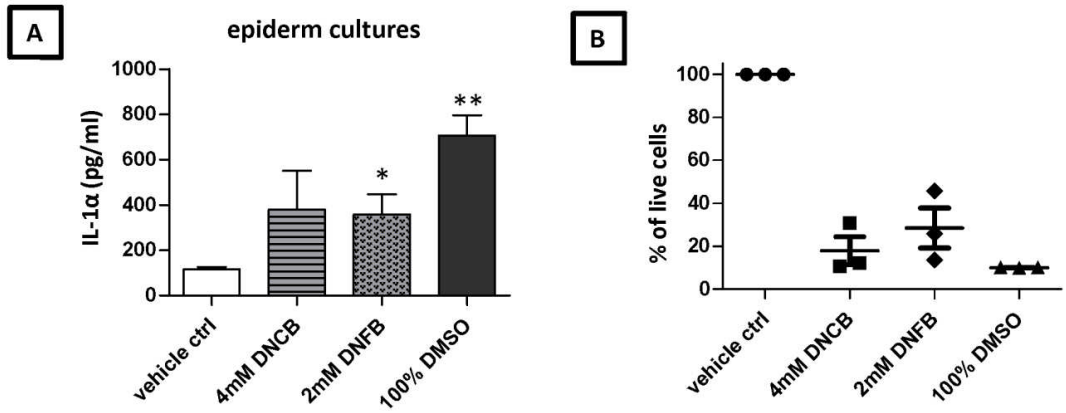


Figure 4-2: Human skin samples release IL-1α after exposure to skin sensitisers and irritants.

EpiDerm™ skin models were treated with sensitisers or irritants for 1 hour and, after washing in PBS, cultured in fresh medium for 24 hours. **A:** The supernatants were collected and analysed for IL-1α release by ELISA. **B:** An MTT assay was performed to determine the amount of cell death. n=3, means+SEM. * $p<0.05$ (paired student's t-test, compared to vehicle ctrl).

4.3.3 DCs produce proinflammatory cytokines in response to HaCaT cell coculture or supernatants from HaCaT cells treated with different chemicals

The supernatants from DCs cocultured with sensitiser-treated HaCaT cells were analysed by Luminex analysis. IL-1α, IL-1RA, IL-6, IL-8, CCL2, CCL3, CCL4 and IP-10 were found in the supernatants (Figure 4-3A). The experimental model did not readily allow us to distinguish which cytokines came from which cell type. However, comparison of Figure 4-1A and Figure 4-3A suggests that DCs exposed to sensitiser/irritant treated HaCaT cells produced IL-8, CCL2 and low levels of CCL3, CCL4 and IP-10 (Figure 4-3A+B). DCs activated with the supernatant of chemically exposed HaCaT cells produced IL-8 and low levels of CCL3 (Figure 4-3A+B).

4.3.4 Neutralisation of IL-1α reduces DC activation induced by chemically treated HaCaT cell supernatant

We were able to show that HaCaT cells, as well as primary KCs, release IL-1α following chemical-induced necrosis (Figure 4-1, Figure 4-2). Furthermore, coculture with such chemical-treated HaCaT cells or their supernatants were sufficient to augment expression of DC maturation markers (Figure 3-8) as well as the release of proinflammatory cytokines and chemokines (Figure 4-3). Therefore, we wanted to investigate whether KC-derived IL-1α is directly involved in DC activation in this model. HaCaT cells were treated with 4mM DNCB, 0.5mM DNFB, 10mM DCNB or 30% DMSO for 1 hour. The chemicals were washed off and the HaCaT cells were subsequently cultured for 20 hours. The supernatants were collected, filtered and stored at -80°C. Thawed supernatants were incubated with an anti-IL-1α antibody or the corresponding isotype control for 30 min, before being added to the DCs for another 20 hours. As shown previously, the supernatant of chemical-treated HaCaT cells was

sufficient to induce upregulation of CD83 and CD86 on DCs, measured by flow cytometry. However, the addition of IL-1α blocking antibodies significantly attenuated CD83 and CD86 expression, showing that KC-released IL-1α directly contributes to DC activation in this model (Figure 4-4). Addition of the isotype control antibody did not attenuate CD83 or CD86 expression induced by the HaCaT supernatants. The block of activation was not complete, suggesting molecules other than IL-1α also contribute to the interaction between the HaCaTs and DCs. Blocking of IL-1α in the supernatants of DMSO-treated HaCaTs seems to have a more profound impact on attenuating DC activation than for the supernatants from other chemicals. This observation suggests that different kinetics or mechanisms leading to necrosis of cells may result in different amounts of IL-1α being released. However, this avenue has not been explored further in this study.

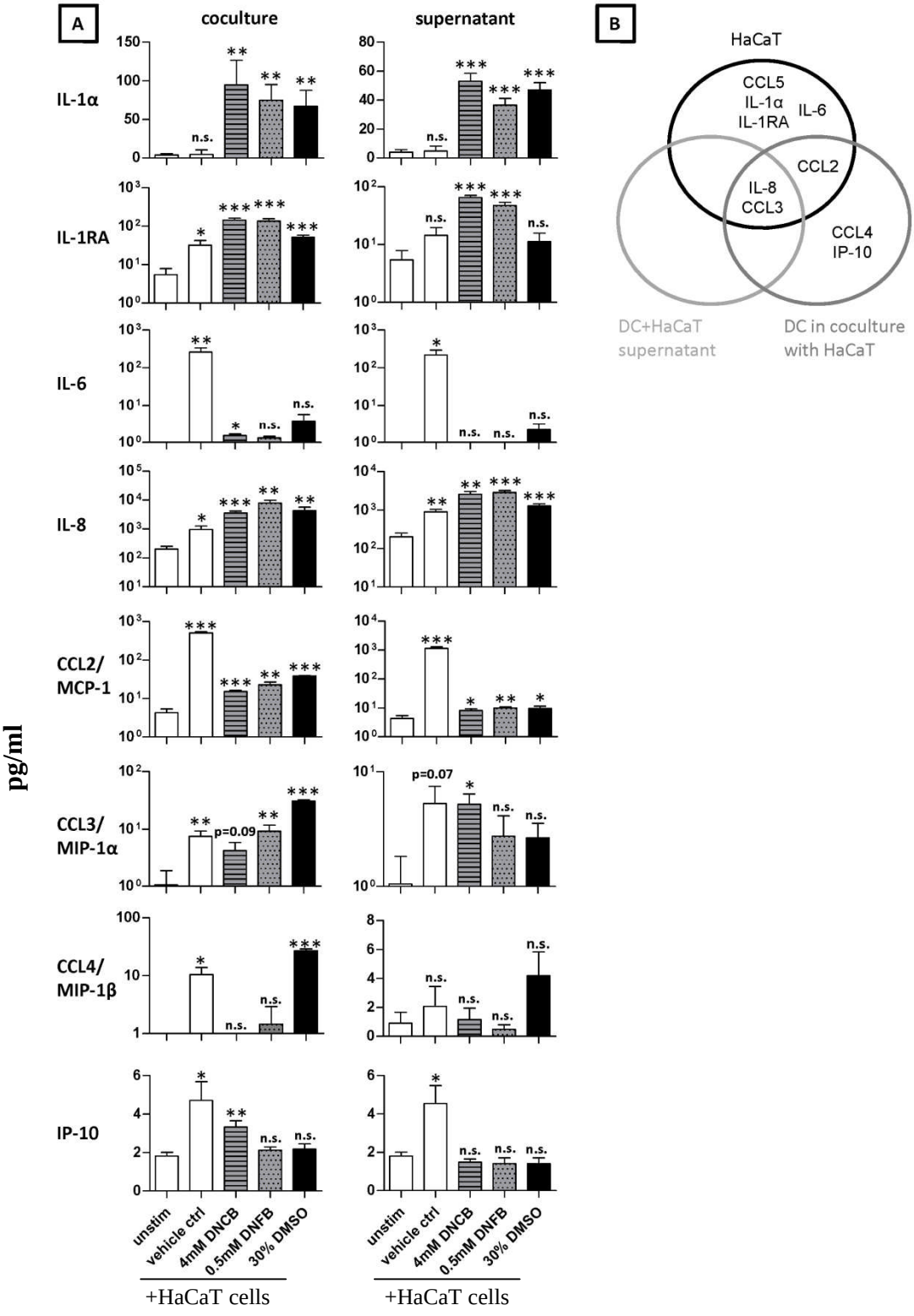


Figure 4-3: Cytokine production of DCs cocultured with sensitiser/irritant-treated HaCaTs or their supernatant.

DCs were cocultured with chemical treated HaCaT cells or HaCaT supernatants for 24 hours. Supernatants were collected and analysed for 25 cytokines/chemokines using a Luminex platform. Results from unstimulated DCs are shown as control. **A:** Representative data showing means of 3-6 experiments+SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (paired student's t-test, compared to unstimulated cells). **B:** Venn-diagram comparing the supernatant from DC cocultured with HaCaT cells, or activated with HaCaT cell supernatant, to the supernatant from chemical treated HaCaT cells (also see Figure 4-1).

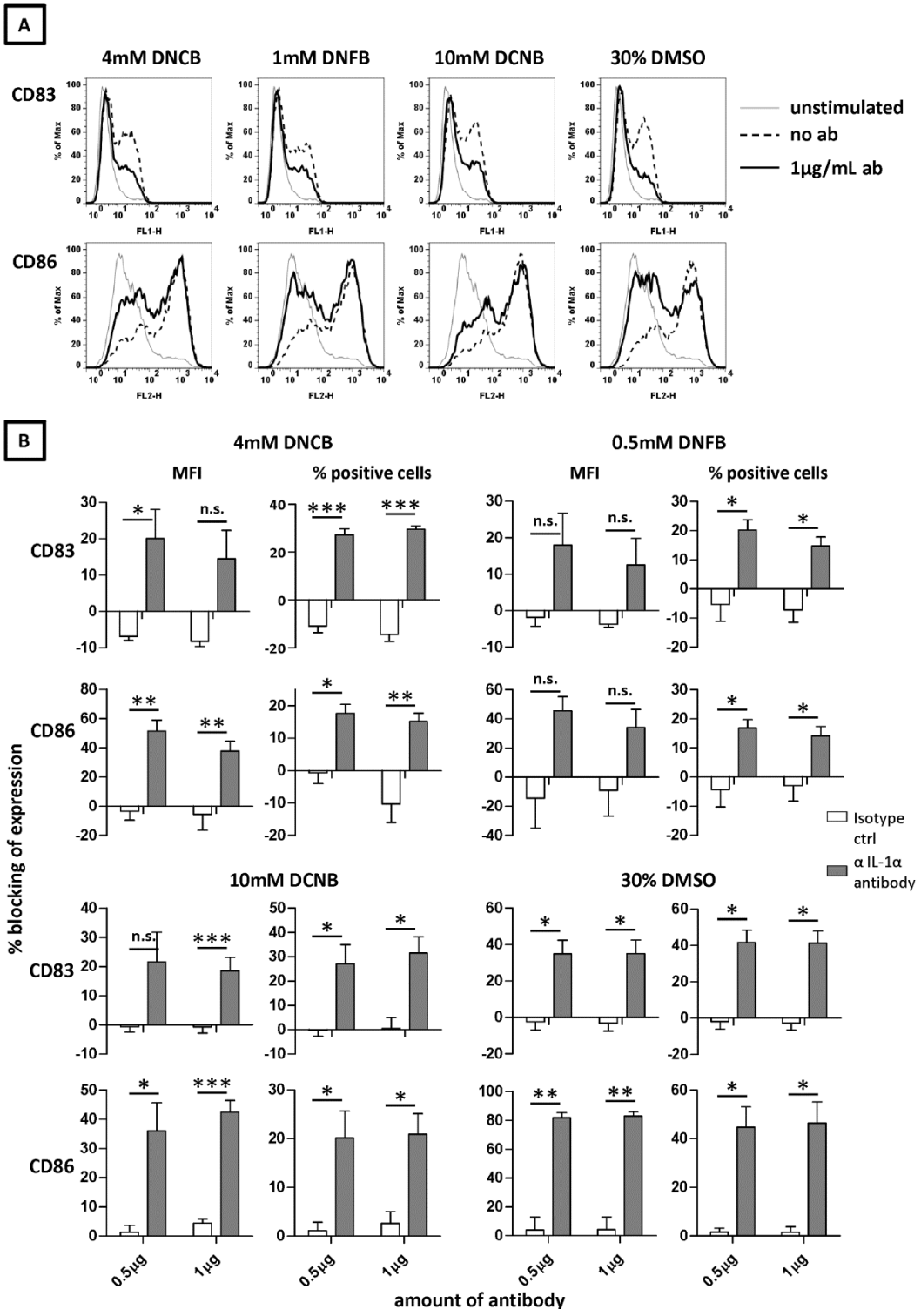


Figure 4-4: Neutralisation of IL-1α reduces DC activation induced by chemical treated HaCaT cell supernatant.

HaCaT cells were treated with the different chemicals for 1 hour. The chemicals were washed off and HaCaT cells were cultured for 24 hours. Supernatants were treated with αIL-1α or isotype control antibody for 30 min before being used to stimulate DC for 20 hours. CD83 and CD86 expression was assessed by flow cytometry. The percentage of positive cells and the median fluorescent intensity were used to calculate the percentage of blocking compared to DC stimulated with supernatant without antibody treatment. **A:** FACS-plot of one representative experiment. **B:** means+SEM of 2-4 experiments, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (paired student's t-test).

4.3.5 Allogeneic T cell responses

Next, we wanted to investigate whether LC/DC exposure to chemically-treated HaCaT cells can result in altered T cell responses. Mixed leukocyte reactions using LCs or DCs as APCs and allogeneic T cells as responder cells provide a useful model to study quantitative differences of stimulated DCs resulting in differential T cell proliferation and/or Th subtype polarisation.

First, we used monocyte-derived LCs, which were stimulated with Poly I:C as a positive control or cocultured with DNFB-treated or necrotic (freeze-thawed) HaCaT cells over night. The LCs were separated from the dead HaCaT cells through density gradient centrifugation and afterwards cocultured with allogeneic T cells, depleted with CD14 (monocytes), CD19 (B cells), CD56 (NK cells) and HLA-DR (activated memory T cells and APCs) antibodies at increasing DC:T cell ratios. T cell proliferation was measured after 3 days, by adding radioactive thymidine for the last 18 hours of the coculture. T cell proliferation was found to be proportional to the number of DCs, even though considerable variability was evident between different allogeneic cultures (Figure 4-5). Repeated Measures (RM) 2-way ANOVA was used to test the effect of innate immune priming and of the number of DCs within matched DC-T cell allogeneic pairs. As expected, there was a highly significant effect of DC numbers ($p<0.0001$) on T cell proliferation. However, neither proliferation (Figure 4-5) nor IFN-γ production (Figure 4-6) of the T cells was significantly altered by LCs exposed to chemically treated HaCaT cells. However, a trend towards augmented proliferation following PolyI:C, DNFB or DMSO treatment was noted. It has been reported previously that LCs induce lower allogeneic T cell responses than DCs (A. Reske's thesis), so we decided to use monocyte-derived DCs instead of LCs. Furthermore, we only decided to use $CD4^+$ T cells as they are the main drivers of allogeneic responses. Indeed, the proliferative

response was augmented using DCs as APCs opposed to LCs. Interestingly, DCs exposed to DNFB-treated HaCaT cells attenuated T cell proliferation (Figure 4-7) and IFN-γ production (Figure 4-8), but did not influence IL-13 (Figure 4-9) or IL-17 release (Figure 4-10). DCs activated with the supernatant of DNFB-treated HaCaT cells did not affect allogeneic T cell responses (Figure 4-7 - Figure 4-10). However, DCs activated with DMSO-treated HaCaT cells or their supernatant augmented T cell proliferation (Figure 4-7), IFN-γ (Figure 4-8) and IL-13 (Figure 4-9) production. IL-17 production was increased only after DCs were cocultured with DMSO-treated HaCaT cells (Figure 4-10).

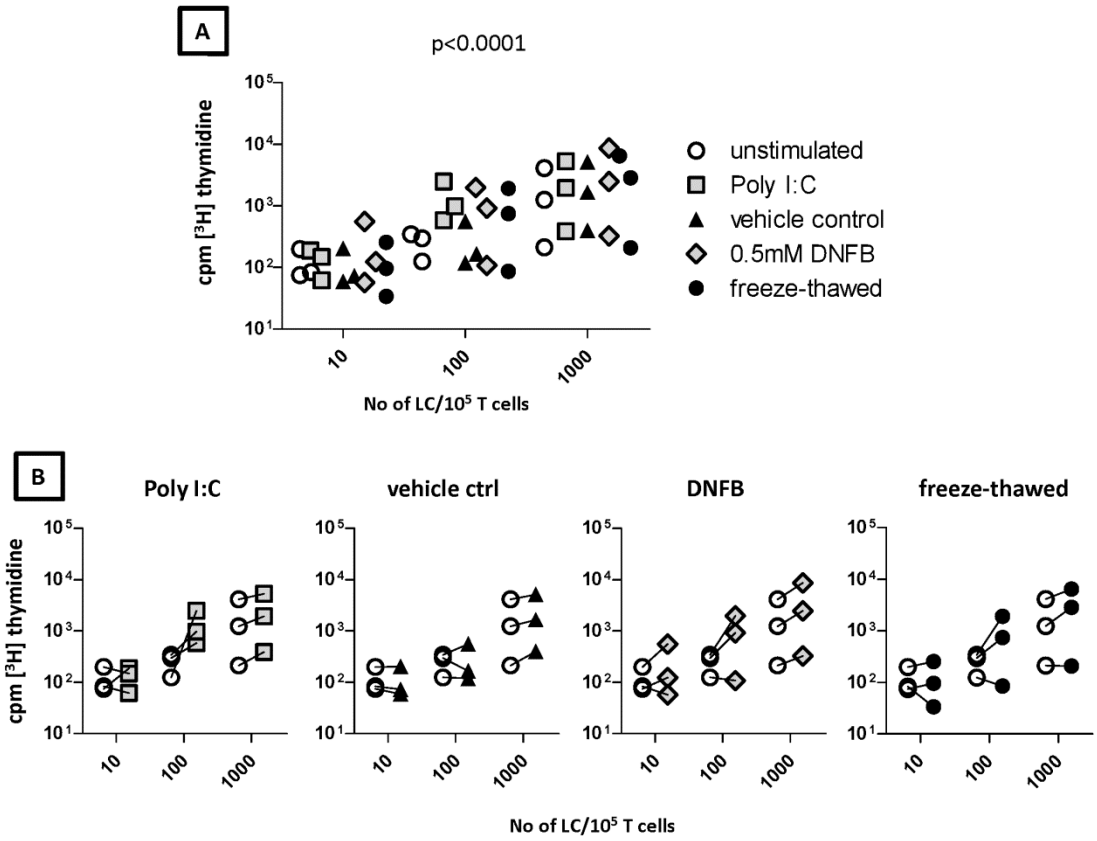


Figure 4-5: T cell proliferation following coculture with allogeneic LCs

LCs were left **unstimulated**, stimulated with 50µg/ml **Poly I:C** or cocultured with **vehicle control**, **0.5mM DNFB**-labelled or **freeze-thawed**-killed HaCaT cells. After 20 hours LC-HaCaT cocultures (ratio of 1 LC : 50 HaCaT cells) were purified through lymphoprep and the live cells were cocultured with allogeneic T cells ($CD14^-$, $CD19^-$, $HLA\text{-}DR^-$, $CD2^-$, $CD56^-$). T cell proliferation was assessed by [^{3}H] thymidine incorporation (counts per minute-cpm) after 3 days. Each data set represents individual DC-T cell pairing. Repeated measure (RM) 2-way ANOVA was used to analyse data. Increasing DC:T cell ratios were associated with significantly increased T cell proliferation **(A)**.

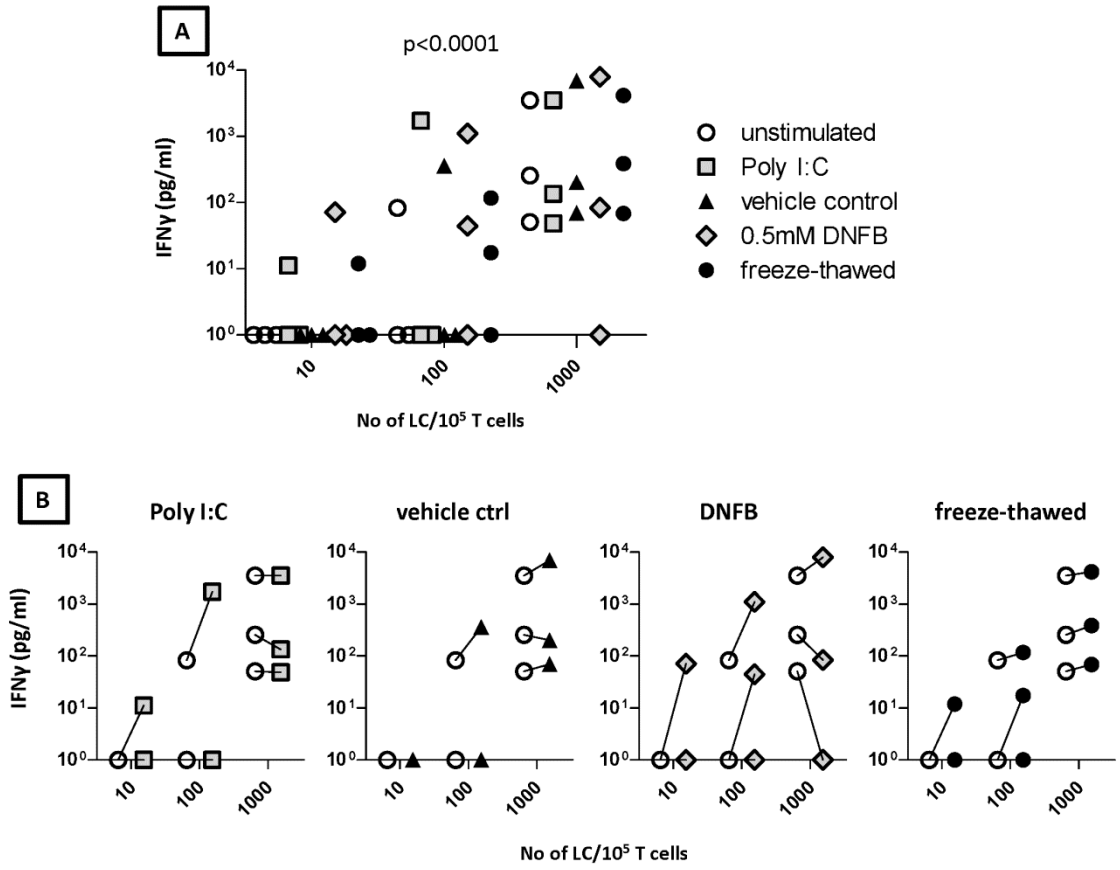


Figure 4-6: IFNγ production by T cells following coculture with allogeneic LCs

LCs were left **unstimulated**, stimulated with 50µg/ml **Poly I:C** or cocultured with **vehicle control**, **0.5mM DNFB**-labelled or **freeze-thawed**-killed HaCaT cells at a ratio of 1 LC : 50 HaCaT cells. After 20 hours LC-HaCaT cocultures were purified through lymphoprep and the live cells were cocultured with allogeneic T cells ($CD14^-$, $CD19^-$, $HLA\text{-}DR^-$, $CD2^-$, $CD56^-$). After 5 days supernatants were collected and subsequently analysed for IFNγ production by ELISA. Each data set represents individual DC-T cell pairing. Repeated measure (RM) 2-way ANOVA was used to analyse data. Increasing DC:T cell ratios were associated with significantly increased IFNγ production **(A)**.

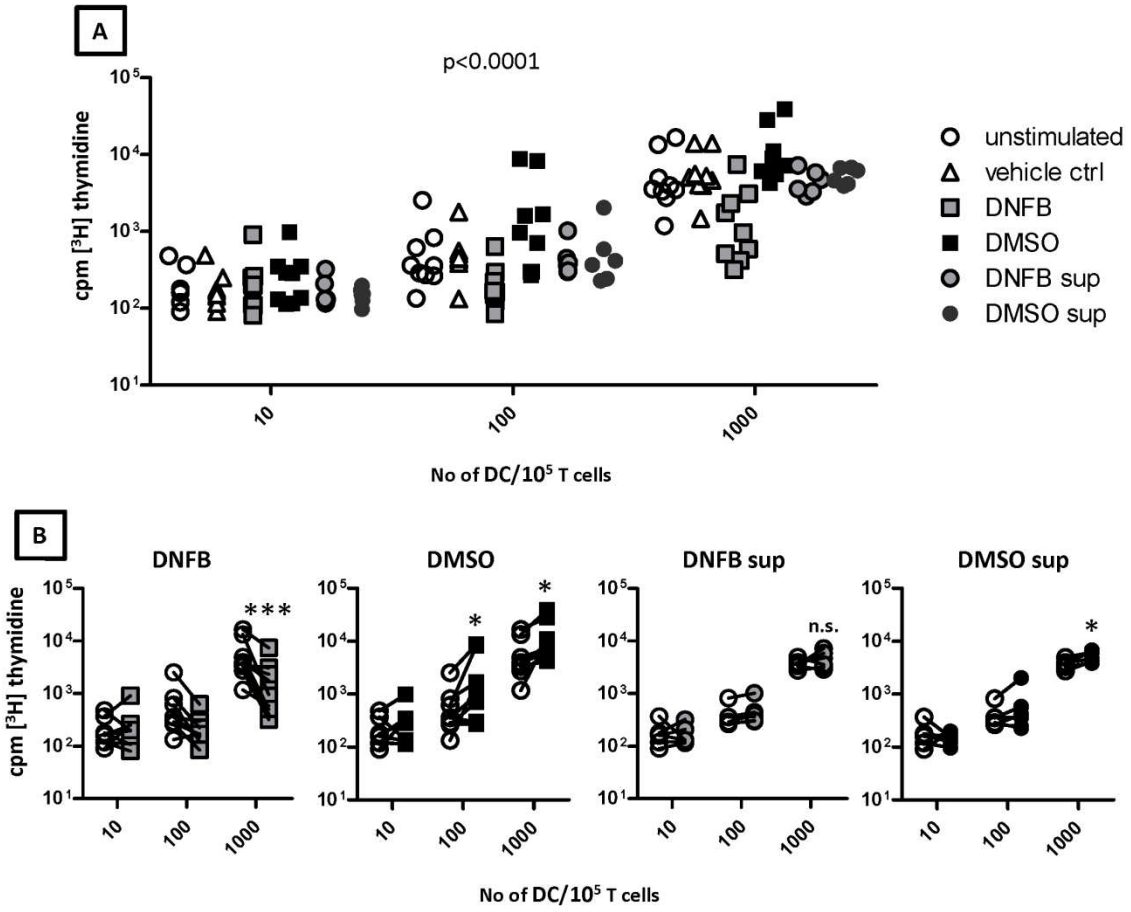


Figure 4-7: CD4$^+$ T cell proliferation following coculture with allogeneic DCs

CD4$^+$ T cell proliferation was assessed by [^{3}H] thymidine incorporation (counts per minute-cpm) after 3 days of coculture with allogeneic DCs. DCs had been activated through coculture with chemical-treated HaCaT cells (**vehicle ctrl**, 0.5mM **DNFB**, or 30% **DMSO**; ratio 1 DC:50 HaCaT) or their supernatants (**DNFB sup, DMSO sup**). Cocultures of **unstimulated** DCs with allogeneic T cells were used as a control. Each data set represents individual DC-T cell pairing. Repeated measure (RM) 2-way ANOVA was used to analyse data. Increasing DC:T cell ratios were associated with significantly increased T cell proliferation **(A)**. RM 2-way ANOVA was used to assess the influence of the DC priming on the T cell proliferation **(B)**.

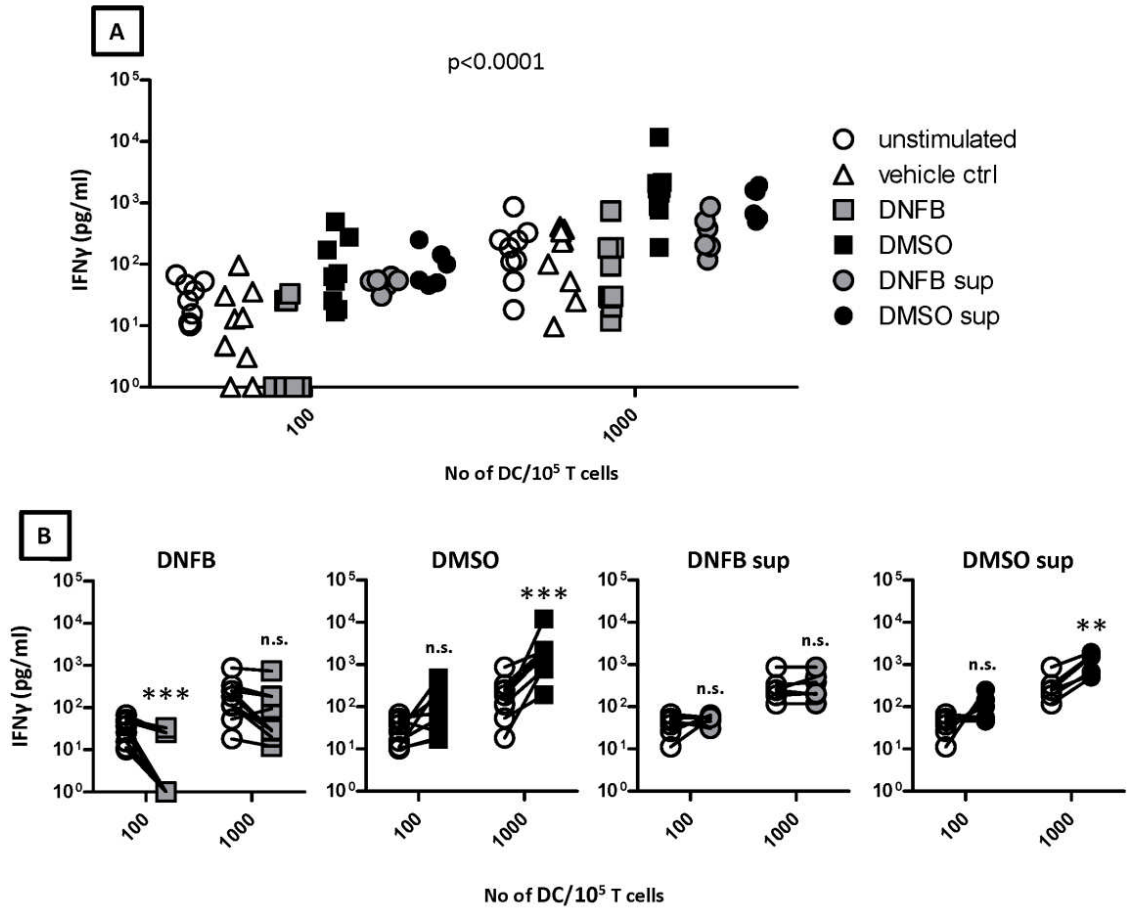


Figure 4-8: IFNγ production by CD4$^+$ T cells following coculture with allogeneic DCs

IFN-γ production of allogeneic DC: CD4$^+$ T cell cocultures after 5 days was assessed by ELISA. DCs had been activated through coculture with chemical-treated HaCaT cells (**vehicle ctrl**, 0.5mM **DNFB**, or 30% **DMSO**; ratio 1 DC:50 HaCaT) or their supernatants (**DNFB sup, DMSO sup**). Cocultures of **unstimulated** DCs with allogeneic T cells were used as a control. Each data set represents individual DC-T cell pairing. Repeated measure (RM) 2-way ANOVA was used to analyse data. Increasing DC:T cell ratios were associated with significantly increased IFN-γ production **(A)**. RM 2-way ANOVA was used to assess the influence of the DC priming on the IFN-γ production **(B)**.

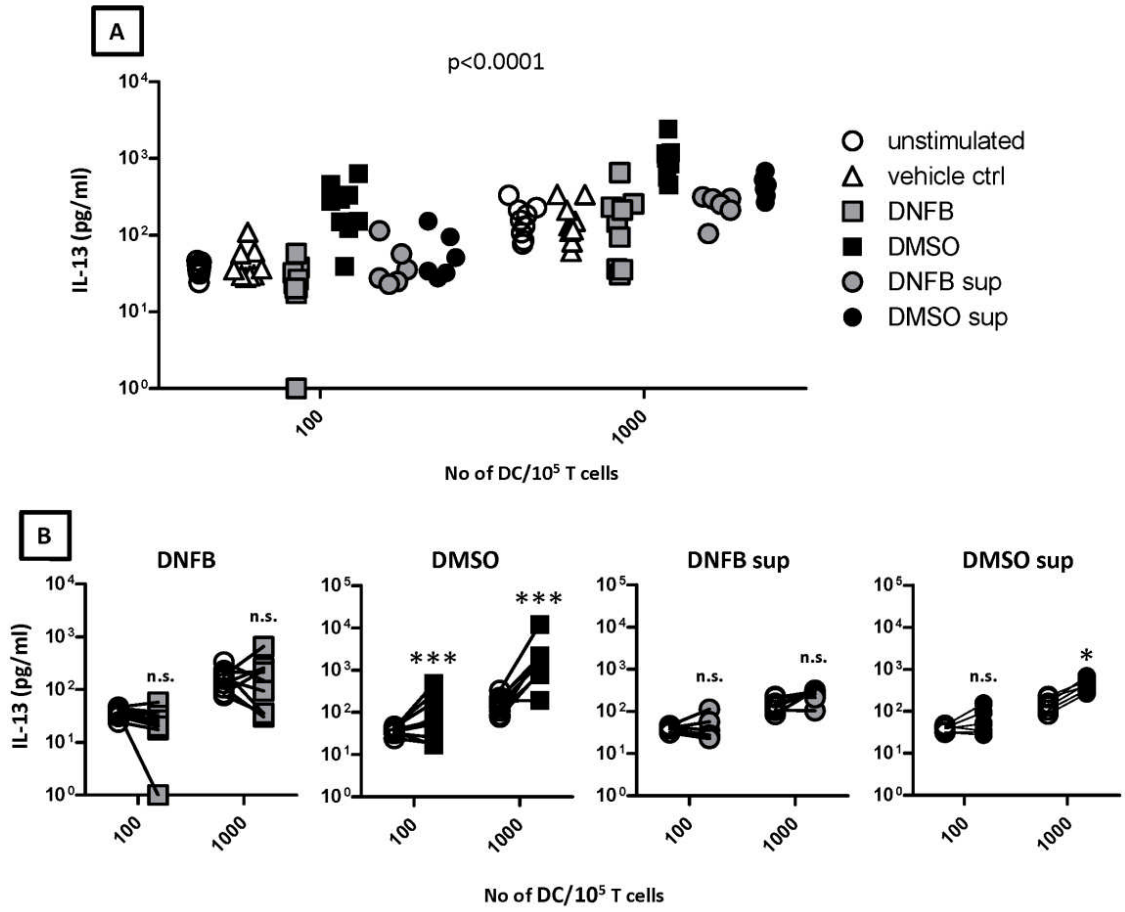


Figure 4-9: IL-13 production by $CD4^+$ T cells following coculture with allogeneic DCs

IL-13 production of allogeneic DC: $CD4^+$ T cell cocultures after 5 days was assessed by ELISA. DCs had been activated through coculture with chemical-treated HaCaT cells (**vehicle ctrl**, 0.5mM **DNFB**, or 30% **DMSO**; ratio 1 DC:50 HaCaT) or their supernatants (**DNFB sup, DMSO sup**). Cocultures of **unstimulated** DCs with allogeneic T cells were used as a control. Each data set represents individual DC-T cell pairing. Repeated measure (RM) 2-way ANOVA was used to analyse data. Increasing DC:T cell ratios were associated with significantly increased IFN-γ production **(A)**. RM 2-way ANOVA was used to assess the influence of the DC priming on the IL-13 production **(B)**.

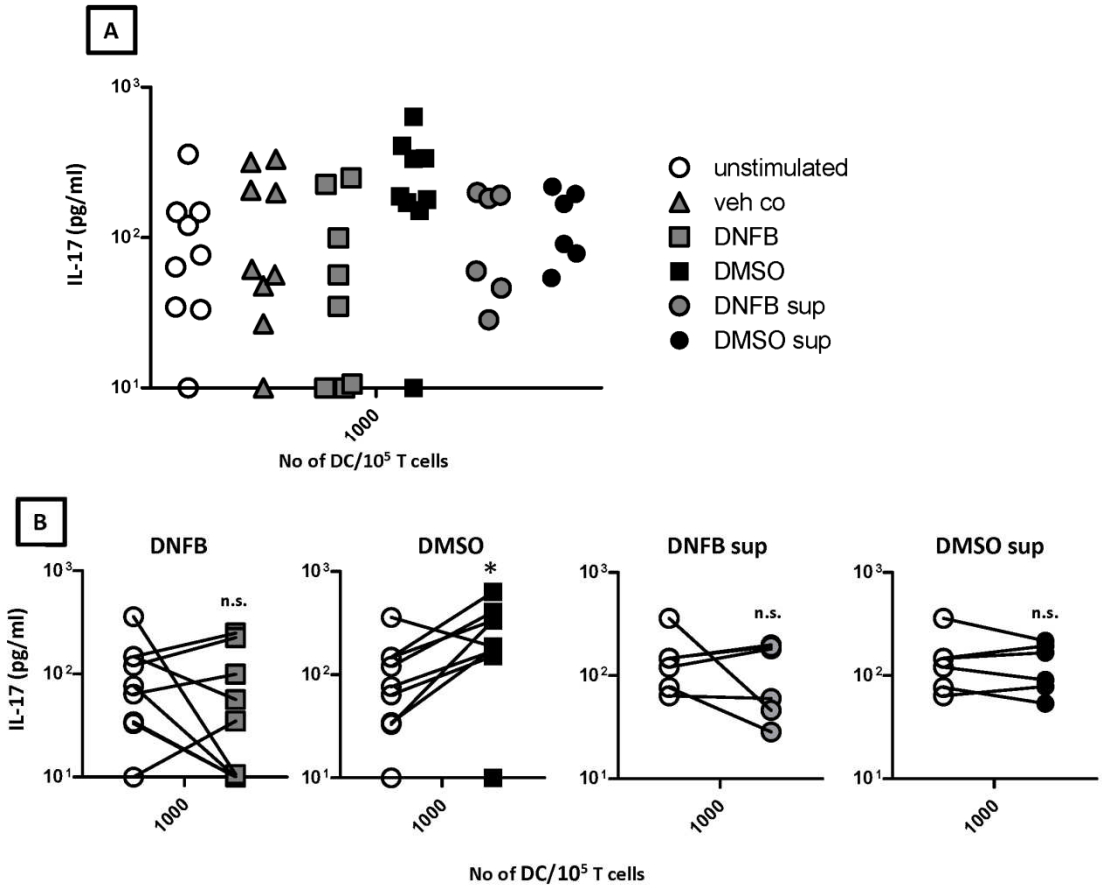


Figure 4-10: IL-17 production by CD4$^+$ T cells following coculture with allogeneic DCs

IL-17 production of allogeneic DC: CD4$^+$ T cell cocultures after 5 days was assessed by ELISA. DCs had been activated through coculture with chemical-treated HaCaT cells (**vehicle ctrl**, 0.5mM **DNFB**, or 30% **DMSO**; ratio 1 DC:50 HaCaT) or their supernatants (**DNFB sup, DMSO sup**). Cocultures of **unstimulated** DCs with allogeneic T cells were used as a control. Each data set represents individual DC-T cell pairing. Paired student´s t-test was used to assess the influence of the DC priming on the IL-17 production **(B)**.

4.4 Discussion

4.4.1 Danger signals released by necrotic cells

Common irritancy alarm signals are thought to play a role in both ACD and ICD. The immunologic response in these two diseases is very divergent. ICD leads to an unspecific inflammation mediated by innate immune cells in the area of insult. In contrast to ACD, no adaptive immune response is initiated and therefore no memory response can be manifested. However, activation and egress of LCs from the epidermis

following chemical application is seen both with ACD and ICD (Cumberbatch et al., 1993; Willis et al., 1990). Cytotoxic effects of these chemicals might result in the release of normally in-the-cell sequestered danger signals. Indeed, we found that chemical-treated KCs released the danger molecule HMGB-1, as well as IL-1α and IL-1RA. Some nice studies by Scaffidi et al., and Cohen et al., showed that HMGB-1 and IL-1α were retained tightly bound to chromatin during apoptosis, even when the cells were subjected to necrosis afterwards, and were only passively released during primary cell necrosis (Cohen et al., 2010; Scaffidi et al., 2002). HMGB-1 has been reported to activate DCs, possibly via different receptors and has as well been implicated as playing a role during sepsis. No blocking antibodies against HMGB-1 are available to date, and we did not study its´ role in DC activation further in our model.

IL-1α has been previously implicated in cutaneous host defence. KCs are known to store large quantities of pro-IL-1α, which can leak out following toxic insult (Corradi et al., 1995; Gahring et al., 1985; Kupper and Groves, 1995; Newby et al., 2000). IL-1α is then thought to stimulate neighbouring KCs and underlying fibroblasts enabling them to release several proinflammatory cytokines and chemokines to attract cells of the innate and adaptive immune system (Bashir et al., 2009; Gahring et al., 1984; Larsen et al., 1989). However, the role of IL-1α in the direct activation of cells of the innate immune system (DC/LC) has not been very well examined. We confirmed that the IL-1α release following sensitiser and irritant treatment was not only a phenomenon of the HaCaT cells by using the epidermis model EpiDerm^{TM}. The EpiDerm^{TM} models consist of primary human KCs which stratify and form an artificial epidermis exhibiting the typical layers normally found in the skin. IL-1α release from EpiDerm^{TM} models following irritant treatment has been shown before as the model had been tested to be a

standard method used for the assessment of the irritancy potential of different chemicals (Spielmann et al., 2007).

The only chemical for which induced cytotoxicity of the HaCaT cells did not correlate with increased DC activation was SDS. Interestingly, we were not able to detect any IL-1α in the supernatants of SDS treated HaCaT cells, suggesting that the lack of IL-1α and possibly other danger signals led to attenuated DC activation.

4.4.2 Activation of DC by danger signals

The danger hypothesis suggests that sentinels, such as DCs, can sense danger resulting in their activation. They can subsequently "report" this danger to cells of the adaptive immune system that become activated when the right antigenic signals are presented. The danger molecule HMGB-1 has been suggested to bind to RAGE, and might additionally be able to signal through various TLRs (Dumitriu et al., 2007; Tian et al., 2007; Yanai et al., 2009; Yang et al., 2010; Yu et al., 2006). It has been suggested that IL-1 can stimulate DCs: however the literature is very old and most studies were done in the murine system. Here, we show for the first time that IL-1α released by moribund KCs contributes to the activation of human mo-DCs. Neutralisation of IL-1α by blocking antibodies did not inhibit DC activation completely, suggesting that further danger signals, such as HMGB-1 and possibly others contribute to DC activation in this model. Because the DC response to IL-1α has not been investigated very thoroughly to date, we studied it further (chapter 5).

Furthermore, we show that DCs cocultured with chemical-treated HaCaT cells or their supernatant produce proinflammatory cytokines and chemokines, such as IL-8, CCL2, CCL3, CCL4 and IP-10. Similar to the expression of the DC maturation markers CD83 and CD86, following coculture the DCs were more activated and the production

of chemokines was augmented compared to DCs treated with the supernatants. Furthermore, it has to be noted that the DCs were cocultured with HaCaT cells in a ratio of 1:50. Therefore the high numbers of the HaCaTs might have masked some of the cytokines which they produced, and secondly some cytokines/chemokines might have been below the detection limit because the DCs were very diluted ($2.5x10^4$/ml).

4.4.3 The role of IL-1α in CHS

As mentioned in chapter 1.3.6, the role of IL-1α during CHS reactions has not been fully elucidated yet. Using a mouse model, it was suggested that IL-1β plays a crucial role in the induction of LC migration after sensitiser treatment, whereas IL-1α is needed for LC egress after irritant exposure (Cumberbatch et al., 2002). However, it has been shown in different murine or human KC cell lines or primary cells, that KCs have the ability to release IL-1α following sensitiser treatment (Corsini et al., 1998; Enk and Katz, 1992; Little et al., 1998; Newby et al., 2000; Spiekstra et al., 2005). Concentrations of the different chemicals as well as the amount of cytotoxicity which is induced are very likely to determine the signals involved in a cutaneous immune response. Additional data from ACD patients might clarify the role of IL-1α in humans. It has been suggested that IL-1 expression is differentially regulated in mice and humans, in that IL-1α is more readily secreted by murine cells, than human cells (Dinarello, 1996). It seems to be generally accepted, however, that additionally to the formation of neo-antigens, danger signals need to be present in order to achieve sensitisation (Martin et al., 2011). Danger-signal induced activation of LCs/DCs is crucial for their migration to lymph nodes and the presentation of the antigens to T cells. Such danger signals can be delivered in multiple ways: (1) as discussed in this thesis, cytotoxicity induced release of IL-1α from KCs can induce DC maturation via IL-1RI binding. (2) Nickel was the first sensitiser to be shown to have the ability to

directly bind human TLR4 at two nonconserved histidines (Schmidt et al., 2010). Furthermore, concomitant absence of TLR2 and TLR4 has been reported to result in resistance to CHS in mice (Martin et al., 2008). Components of the extracellular matrix might become endogenous ligands for TLR2/4 (Martin et al., 2011). (3) Contact sensitisers might be able to trigger DC activation by modifying thiol groups on the cell surface (Bruchhausen et al., 2003; Kagatani et al., 2010) or inducing an antioxidant response, by activating the Keap1/Nrf2 pathway (Natsch, 2010). (4) Furthermore, it was shown in mice that the inflammasome plays an important role during the sensitisation phase of CHS (Sutterwala et al., 2006; Watanabe et al., 2007).

4.4.4 Allogeneic T cell responses

In order to further study the functionality of the DCs cocultured with sensitiser-/irritant-treated HaCaTs or their supernatants, we cultured them with allogeneic T cells and assessed the T cell proliferation and production of cytokines.

As expected, we found that increasing numbers of DCs in mixed leukocyte reactions induced augmented T cell responses (proliferation and IFN-γ production) (Banchereau and Steinman, 1998; Felix and Allen, 2007; Le Bert et al., 2011; Pollara et al., 2003).

Interestingly, we found that DCs cocultured with DMSO-treated HaCaTs or their supernatants augmented allogeneic T cell reactions (proliferation and IFN-γ production), while in contrast DCs cocultured with DNFB-treated HaCaT attenuated the T cell responses. DCs stimulated with the supernatant of DNFB-treated HaCaT cells did not alter the T cell responses significantly. This came as a surprise, as in line with the hypothesis that innate immune responses increase adaptive immunity, DC activation usually translates into augmented T cell proliferation in mixed leukocyte reactions

compared to unstimulated DCs. It has been suggested before, that in order to obtain sensitiser-specific T cell responses the regulatory T cells had to be removed (Vocanson et al., 2008). It is not clear however, how the DCs treated with DNFB-HaCaT could have specifically activated the Treg population. Alternatively DCs in culture might have become dysfunctional ("exhausted") by phagocytosing the moribund KCs. However, DMSO-treated HaCaTs were also phagocytosed by DC. The inhibitory action of DNCB-treated KCs warrants further study.

4.5 Summary

- KCs killed through sensitiser or irritant treatment release danger signals, such as IL-1α and HMGB-1
- IL-1α released by moribund KCs can activate DCs
- DCs activated by the supernatant of chemically-killed KCs produce proinflammatory cytokines
- DCs cocultured with DMSO-treated KCs augment allogeneic T cell responses, whereas DCs cocultured with DNFB-treated KCs suppress allogeneic T cell responses

5 MODULATION OF DC RESPONSES BY IL-1A

5.1 Introduction

Dendritic cells reside in the skin acting as sentinels to protect the skin. They are placed strategically at the interface to the outside environment ready to pick up antigen of any invading substance. They can be activated by recognising PAMPs of invading microbes or DAMPs induced by pathogens or mechanical or chemical insult. We have shown in the last 2 chapters that chemical sensitisers or irritants can induce necrosis in KCs, the major cell type in the epidermis. KCs are known to store large amounts of the IL-1α precursor, which is released during necrosis. Furthermore, we were able to show that IL-1α released from moribund KCs can act as a danger signal and contribute to DC activation. This might play an important role to ensure immunosurveillance in the skin. The activation of DCs by IL-1α has not been well investigated thus far, so we thought to dissect this response further by using genome-wide transcriptional profiling and comparing the response to other TLR ligands.

5.2 Objectives

- Investigate if IL-1α leads to DC maturation and compare to LPS.
- Study the transcriptional profile of DCs after IL-1α stimulation and compare to LPS and Pam_3CSK4. Investigate if the stimuli lead to similar or different changes in the transcriptome and identify possible pathways.
- Study the cytokines/chemokines released by IL-1 stimulated DC and compare to LPS stimulated DC.
- Study the allogeneic T cell responses induced by IL-1α or LPS stimulated DC.

5.3 Results

5.3.1 IL-1α upregulates DC maturation markers

Our data from the previous chapter suggested that IL-1α released from moribund KCs plays a role in DC activation. In order to investigate the role of IL-1α in DC maturation further, we stimulated immature DCs with increasing concentrations of IL-1α. This led to an increased proportion of cells expressing CD83 and CD86 measured by flow cytometry after a 24 hour stimulation period (Figure 5-1A). 40-50% of the cells expressed CD83 and CD86 at the saturating IL-1α concentration of 1 ng/ml – 10 ng/ml (Figure 5-1A). Cells were either found to coexpress CD83 and CD86 or not to express the markers at all (Figure 5-2B).

Comparison of different DC maturation markers after IL-1α and LPS stimulation revealed that IL-1α, like LPS, led to an upregulation of CD54 (ICAM-1), MHC class II, the T cell costimulatory molecules CD80 and CD86, the DC activation marker CD83, and the T cell inhibitory molecules PD-L1 and PD-L2 (Figure 5-1B). However, either the median fluorescent intensity (MFI) was found to be lower or fewer cells responded to IL-1α stimulation compared to LPS treatment. We therefore wondered if the non-responding cells showed an attenuated expression of IL-1RI. Although the MFI for IL-1RI of IL-1α stimulated cells was significantly lower compared to unstimulated cells, we did not find a correlation between the expression of IL-1RI and the activation markers CD83 or CD86 (Figure 5-2A).

5.3.2 IL-1α activated DCs augment allogeneic T cell responses

Next, we investigated the influence of IL-1α activation of DCs on their ability to stimulate allogeneic T cell responses (Figure 5-3). IL-1α stimulated DCs induced increased T cell proliferation compared to unstimulated DCs, in agreement with their

activated phenotype. Activation of DCs with IL-1α also led to an increased release of IFN-γ and IL-13 in the DC-T cell cocultures. The high ratio of IFN-γ to IL-13 suggested a Th1 bias. IL-1α did not alter IL-17 levels.

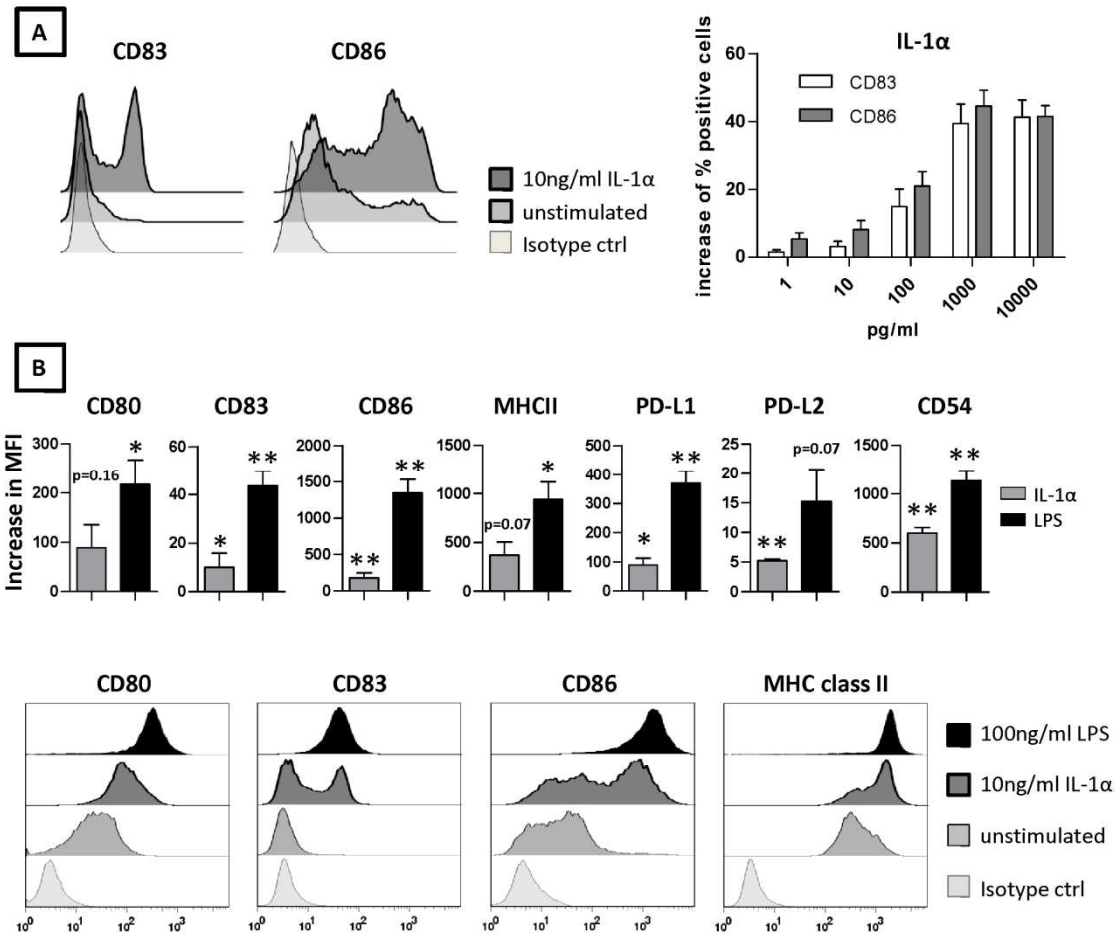


Figure 5-1: IL-1α activates DCs.

Monocyte-derived DCs were stimulated with indicated amounts of IL-1α **(A)** or 10ng/ml IL-1α or 100ng/ml LPS (*E. coli*) **(B)** for 20 hours. Expression of proteins was assessed by flow cytometry. **A:** Increase in CD83 and CD86 expression relative to unstimulated cells following IL-1α treatment is shown (n≥5). **B:** The increase in expression of CD80, CD83, CD86, CD54, MHCII, PD-L1 and PD-L2 relative to unstimulated cells is shown. (n=4). Representative FACS-plots are shown for CD80, CD83, CD86 and MHC class II. Values are presented as means + SEM; n.s. not significant, * $p<0.05$, ** $p<0.01$ (paired student's t-test), MFI = median fluorescent intensity.

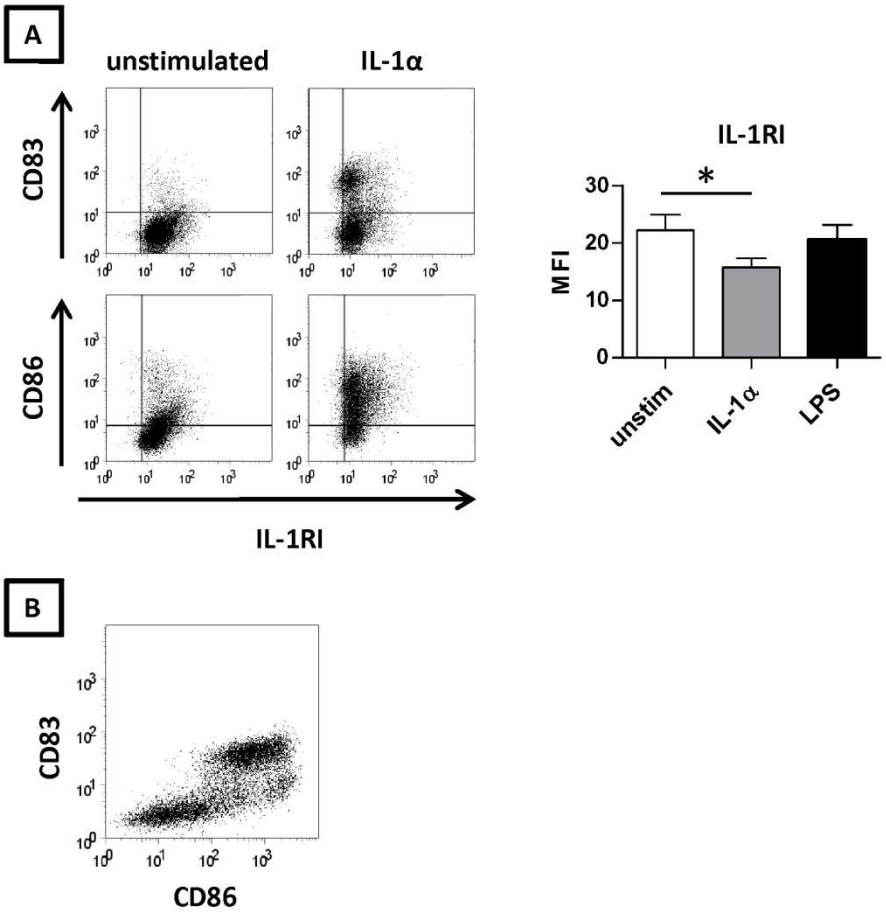


Figure 5-2: IL-1RI is uniformly expressed on DCs.

iDCs were stimulated with 10ng/ml IL-1α or 100ng/ml LPS (*E. coli*) for 20 hours. Cells were stained with the indicated antibodies and analysed by flow cytometry. **A:** Coexpression of IL-1RI and CD83 as well as IL-1RI and CD86 is shown. MFI of IL-1RI expression is shown (n=3). Values are presented as means + SEM; **B:** Coexpression of CD83 and CD86 following IL-1α stimulation (10ng/ml) is shown (one representative experiment of 5). n.s. not significant, * $p<0.05$ (paired student's t-test), MFI = median fluorescent intensity.

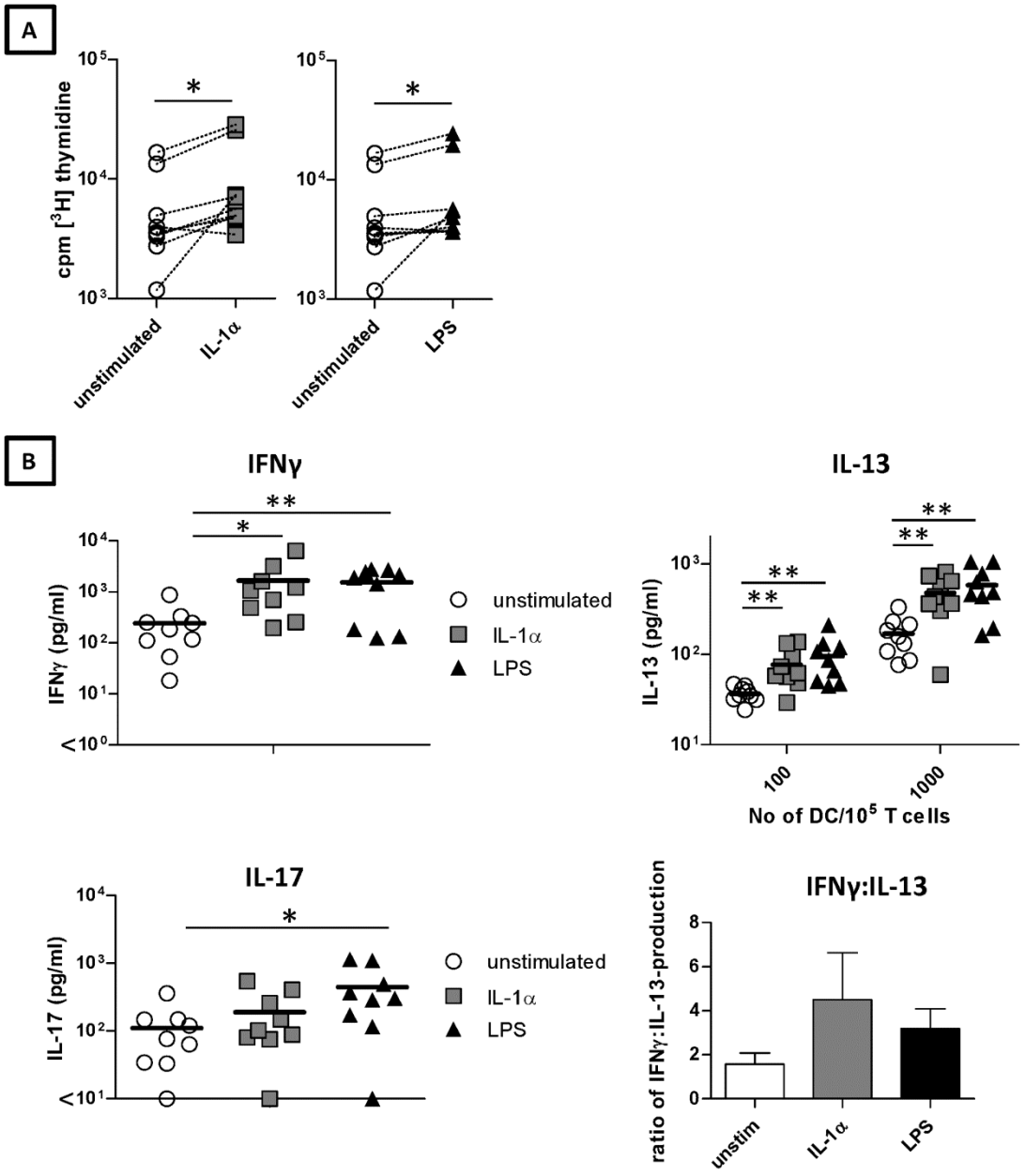


Figure 5-3: IL-1α activated DCs augment allogeneic T cell responses.

DC were stimulated with 10ng/ml IL-1α or 100ng/ml LPS for 20 hours. Afterwards cells were washed and cocultured with $CD4^+$ autologous T cells, at 1000 DC to 10^5 T cells, unless stated otherwise. **A:** T cell proliferation was assessed by thymidine incorporation. **B:** Supernatants were collected after 5 days of coculture and analysed for IFNγ, IL-13 and IL-17 by ELISA. Each data set represents individual DC:T cell experiments. Black line shows the mean. cpm= counts per minute, * $p<0.05$, ** $p<0.01$ (paired student's t-test).

5.3.3 IL-1α induces fewer changes in the transcriptome of DCs compared to LPS

Genome-wide transcriptional profiling using cDNA microarrays provide a useful technique to study overall changes in transcriptional expression in response to different stimuli. RNA of DCs from four different donors stimulated with IL-1α for 4 hours was collected and analysed on an Agilent 4 x 44K whole human genome cDNA microarray. The data were $\log_2$ transformed and LOESS normalised (Chain et al., 2010) to obtain relative expression values that can be compared to former experiments. Gene expression values obtained from IL-1α stimulated DCs were compared to baseline gene expression in DCs from 13 different donors collected over the past six/seven years. Furthermore, transcriptional profiles of LPS and palmitoylated N-acyl-S-diacylglyceryl cysteine-serine-lysine-4 (Pam_3CSK4) stimulated DCs were included in the analysis (Le Bert et al., 2011). Pam_3CSK4 is a synthetic triacylated lipopeptide, which activates TLR2.

Analysing the number of genes that change significantly after stimulation ($p<0.05$; >2-fold change) we found that LPS induces a larger change in the transcriptome compared to IL-1α and Pam_3CSK4 (Figure 5-4A). More than twice as many probes were up- or downregulated (Figure 5-4A+C). However, we found that regardless of the treatment about 1/3 of the genes were upregulated, whereas 2/3 of the genes were downregulated (Figure 5-4A). Comparison of the gene expression profile of IL-1α stimulated DCs with LPS and Pam_3CSK4 stimulated DCs suggested that part of the response to the different stimuli is shared; however, each of the stimuli also induces a unique transcriptional profile (Figure 5-4B+C).

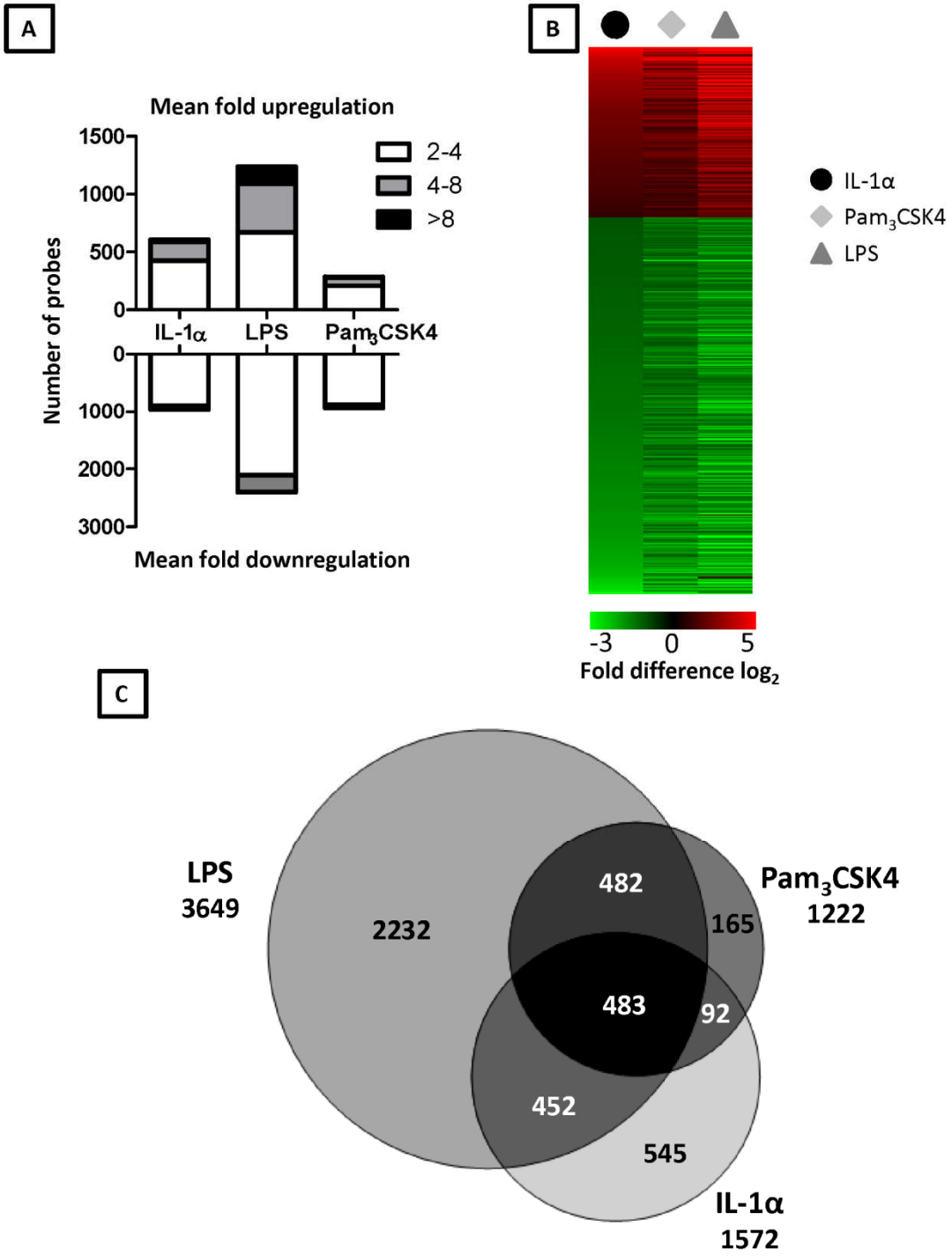


Figure 5-4: IL-1α induces fewer changes to the transcriptome compared to LPS.

A: Quantitive representation of significant up- and downregulation of gene expression in DCs after stimulation with IL-1α (10ng/ml), LPS (100ng/ml, *S. enterica*) and Pam_3CSK4 (1µg/ml) after 4h. **B:** Genes, whose expression changed significantly after IL-1α stimulation were aligned in a heatmap and compared to LPS and Pam_3CSK4 treatment. The mean fold change compared to unstimulated DC is shown. **C:** All significant gene expression differences are compared in a Venn diagram showing the numbers of overlapping or unique genes after stimulation. Data for the stimulated DCs are derived from three or four different experiments using different donors and were compared to unstimulated DCs from 13 different experiments using different donors. Significant changes were defined as changes in expression compared to unstimulated cells (t-test, $p<0.05$) and >2-fold difference. Agilent cDNA microarray gene expression profiling was used to obtain these datasets.

In silico analysis of transcription factor binding motifs was performed on the genes, which were upregulated significantly by each stimulus. Transcription factor (TF) binding sites for the NFκB and ETS family were enriched among the upregulated genes following IL-1α, Pam_3CSK4 and LPS stimulation (Figure 5-5). Upon IL-1α stimulation the predominant transcriptional response seemed to be associated with the NFκB family. Signal Transducers and Activators of Transcription 1 (STAT1), Interferon regulatory factor (IRF)2 and TF of the ETS family seem to play a minor role. STAT1, IRF2, IRF1 and TFs of the NFκB family are enriched to a similarly high degree following LPS stimulation. Transcription factor binding sites for the NFκB family and p53 were enriched in the upregulated genes following Pam_3CSK4 treatment, but neither STAT1 nor IRF1/2 binding sites were significantly enriched. However, the zinc-finger TFs Sp1 and myeloid zinc finger (MZF)1_1-4 were associated with Pam_3CSK4 response, as opposed to the response following IL-1α and LPS stimulation. TF binding analysis therefore confirmed that many of the underlying responses to the innate stimuli IL-1α, LPS and Pam_3PCSK4 are shared; however each of the stimuli also induces a unique response.

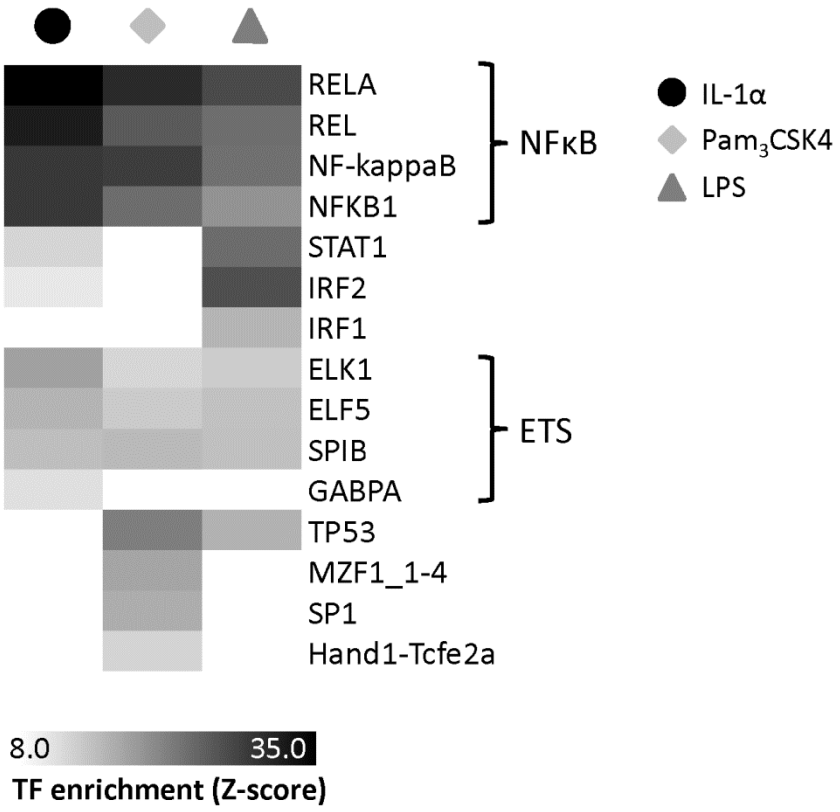


Figure 5-5: The main transcriptional response following IL-1α, Pam_3CSK4 and LPS stimulation is shared.

iDCs were stimulated with IL-1α (10ng/ml), LPS (100ng/ml, *S. enterica*) and Pam_3CSK4 (1µg/ml) for 4 hours. RNA was extracted and samples were analysed by Agilent cDNA microarray gene expression profiling. Enriched transcription factor (TF) binding sites within the significantly upregulated genes were determined using the online tool oPOSSUM. TF binding sites with a Z score higher than 10 are shown, which is usually used as significance cut off. The higher the Z score the more overrepresented are the TF binding sites. Data for the stimulated DCs are derived from three or four different experiments using different donors and were compared to unstimulated DCs from 13 different experiments using different donors. Significant changes were defined as changes in expression compared to unstimulated cells (t-test, $p<0.05$) and >2-fold difference.

5.3.4 IL-1α induces a proinflammatory signature in DC

Functional annotation clustering of the probes significantly up- or downregulated after stimulation revealed that stimulation with either stimulus led to an augmented expression of genes associated with an inflammatory/defence response, regulation of apoptosis, the regulation of signal transduction and cytokine/chemokine activity. Representative gene ontology (GO) terms of the most enriched functionally related clusters can be found in Figure 5-6. The GO terms for the downregulated probes were mainly associated with metabolic and cellular housekeeping processes. We had already observed that IL-1α stimulated DCs adopted a proinflammatory phenotype by augmenting the expression of surface activation and maturation markers, albeit lower than after LPS stimulation (Figure 5-1). These findings are supported by the gene expression data showing that IL-1α stimulation (as well as Pam_3CSK4 stimulation) results in augmented gene expression levels of DC maturation makers, such as CD40, CD54 (ICAM I), CD80, CD83, CD86, CD274 (PD-L1) and CCR7 (Figure 5-7). Moreover, LPS stimulation augmented the expression levels even further consistent with the flow cytometry data (Figure 5-7, Figure 5-1).

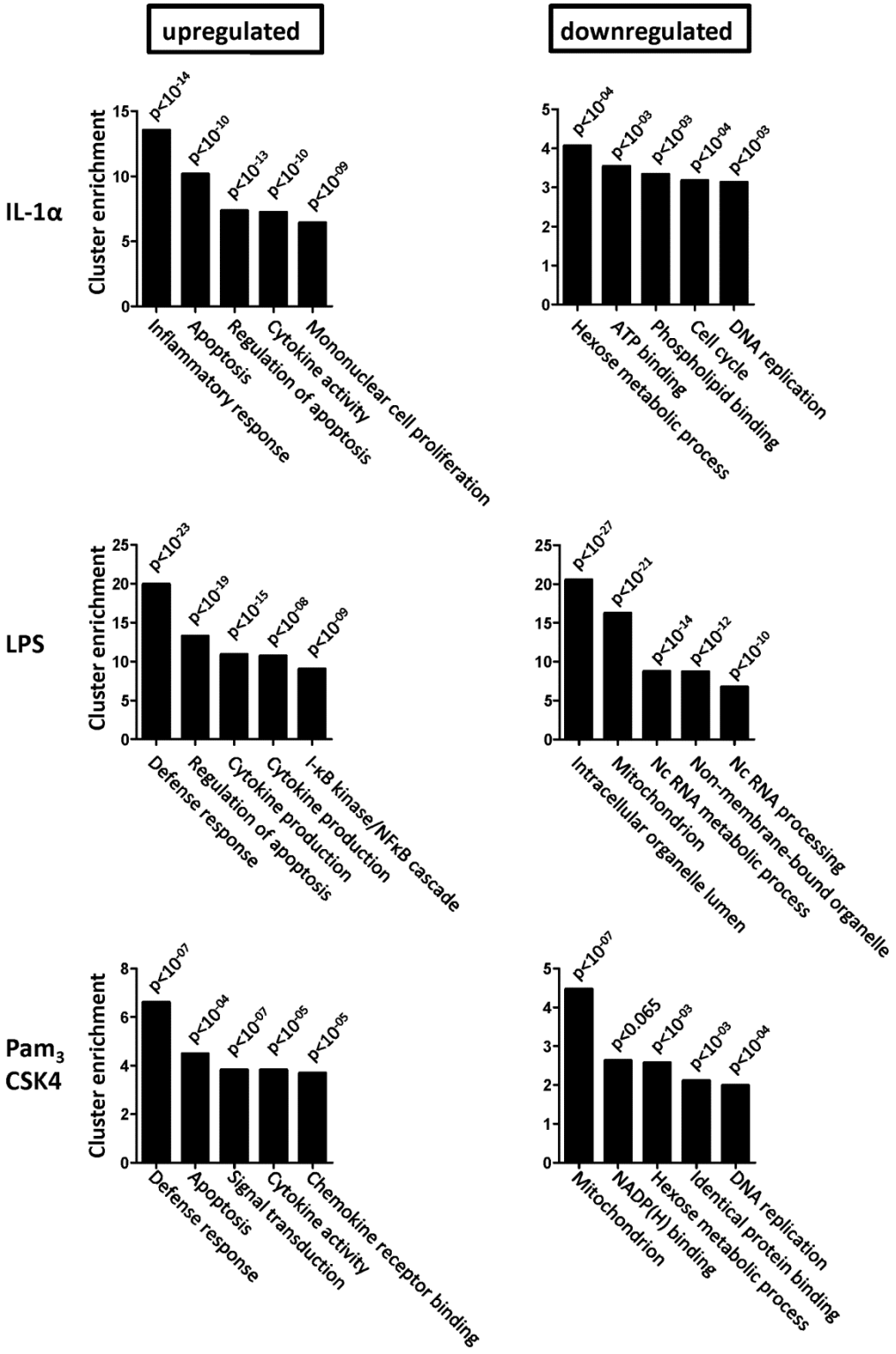


Figure 5-6: Upregulated genes are associated with inflammation, whereby downregulated genes are associated with cellular housekeeping.

iDCs were stimulated with IL-1α (10ng/ml), LPS (100ng/ml, *S. enterica*) and Pam_3CSK4 (1µg/ml) for 4 hours. Samples were analysed by Agilent cDNA microarray gene expression profiling. Representative gene ontology (GO) terms of the top five enriched clusters identified by functional annotation clustering using the online resource DAVID of probes that were significantly ($p<0.05$, >2-fold change) up- or downregulated is shown (Modified Fisher's Exact Test).

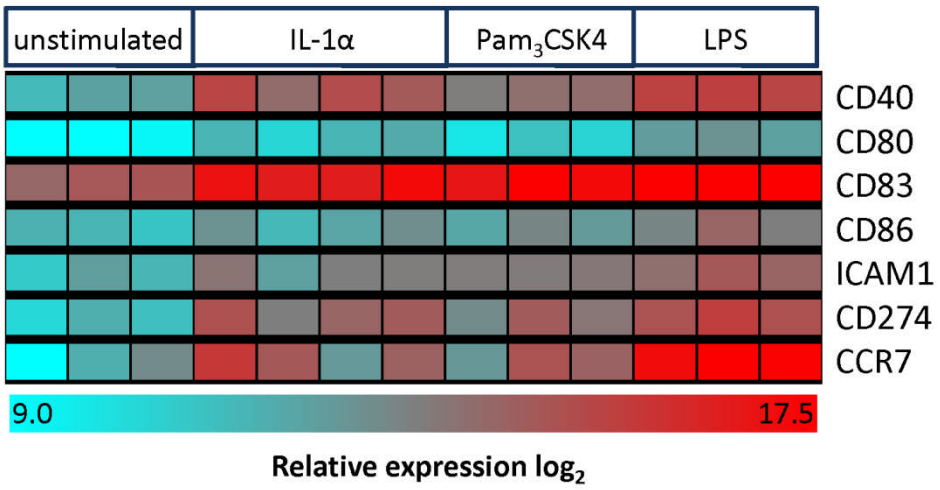


Figure 5-7: Upregulation of dendritic cell maturation markers after stimulation with IL-1α, Pam_3CSK4 and LPS.

iDCs were stimulated with IL-1α (10ng/ml), LPS (100ng/ml, *S. enterica*) and Pam_3CSK4 (1µg/ml) for 4 hours. Samples were analysed by Agilent cDNA microarray gene expression profiling. Relative expression of selected genes associated with DC maturation of 3-4 donors is shown.

Increased expression of maturation markers at the surface of DCs following activation is accompanied by the secretion of cytokines and chemokines, which can modulate immune responses. Functional annotation clustering identified enriched gene clusters related to cytokine activity within the top five enriched clusters following each stimulus (IL-1α, LPS, Pam_3CSK4) (Figure 5-6). Figure 5-8A, showing the gene signatures of most cytokines and chemokines, indicates that the qualitative response following IL-1α and LPS stimulation is comparable, albeit LPS leading to a quantitatively larger response. Pam_3CSK4 seems to induce a more restricted response, however. In order to study the cytokine/chemokines release following IL-1α and LPS stimulation at the protein level a luminex analysis was performed, whereby the release of 25 different cytokines and chemokines was measured in DC supernatants 24 hours after stimulation (Figure 5-9). IL-1α stimulated DCs were found to produce a number of proinflammatory cytokines and chemokines including IL-6, IL-8, IL-12, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CXCL9/MIG, IP-10 and IL-1RA. With the exception of

CCL2/MCP-1, where similar amounts were observed, the IL-1α stimulated DCs produced less of these cytokines/chemokines compared to LPS stimulated cells. Cytokines that were only produced by LPS and not by IL-1α stimulated DCs were TNF-α, IL-1β, IFN-α, IL-2R, IL-4, IL-5, IL7, IL-10, IL-13, IL-15, IL-17 and CCL5/RANTES. Broadly speaking, the luminex data agree with the gene expression data (Figure 5-8B, Figure 5-9) in that IL-1α and LPS induce the production of proinflammatory cytokines and chemokines; however IL-1α leads to a quantitatively smaller response. Some cytokines like IL-1β or TNF-α might have been expected to be released following IL-1α stimulation according to the microarray data, but the amounts might have been below the detection level or posttranscriptional mechanisms might have influenced the release. Furthermore, it needs to be noted that the gene expression data were obtained using LPS from *Salmonella enterica* serotype typhimurium (L6143/045K4056), whereas all other experiments use LPS from *E. coli*. However, in conclusion, IL-1α stimulated DCs show a proinflammatory phenotype like LPS stimulated DCs but they produce a more restricted subset of cytokines and chemokines. This lower overall response might, at least in part, be due to the absence of the IFN feedback loop following IL-1α and Pam_3CSK4 stimulation. Message for IFN-β and release of IFN-α were only found following LPS stimulation (Figure 5-8B, Figure 5-9A), which is in agreement with the absent or very weak signatures of STAT1 and IRF1/2 following IL-1α and Pam_3CSK4 stimulation (Figure 5-5).

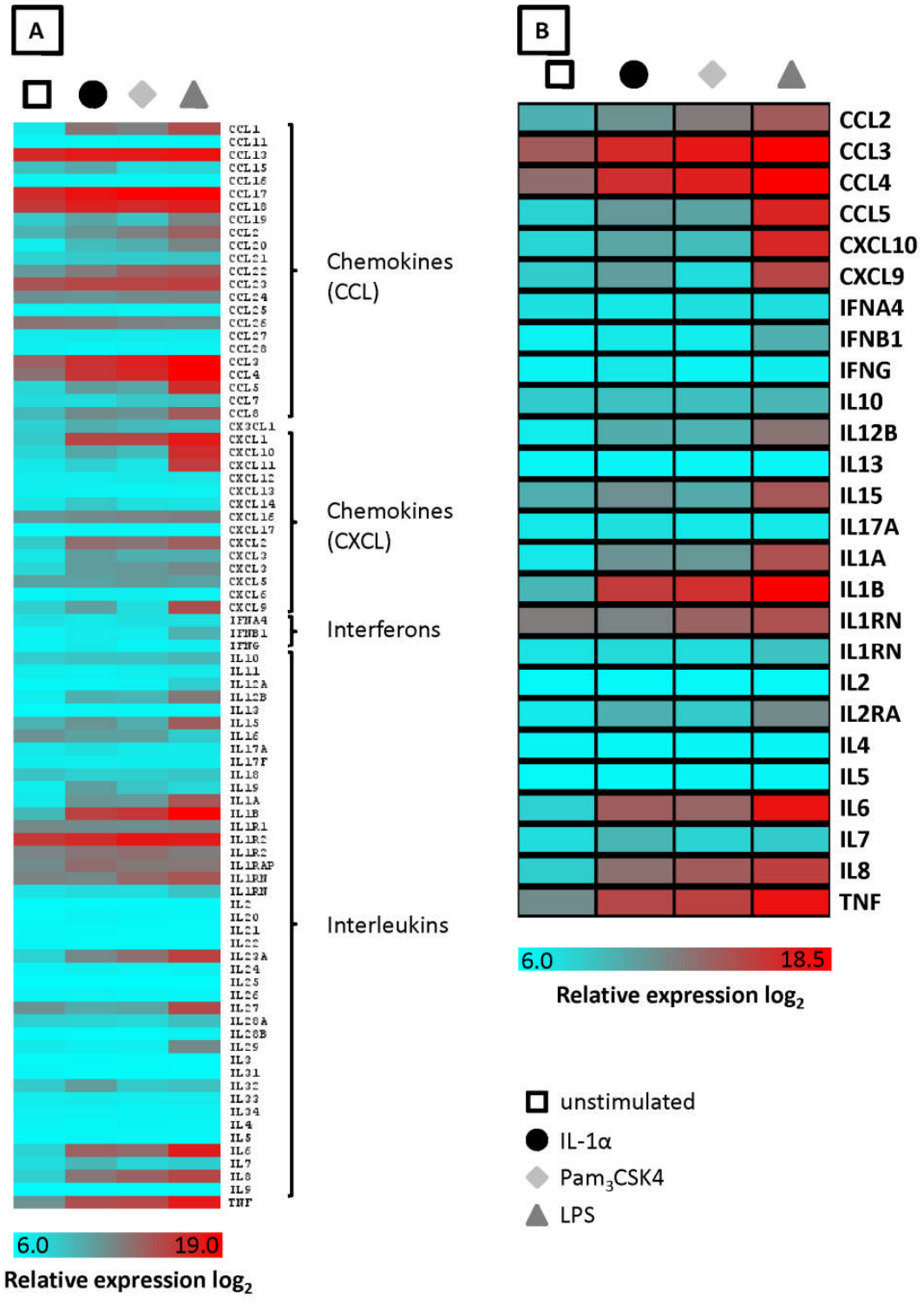


Figure 5-8: IL-1α induces changes in the cytokine/chemokines profile of DC that is quantitatively smaller than after LPS stimulation.

iDCs were stimulated with IL-1α (10ng/ml), LPS (100ng/ml, *S. enterica*) and Pam_3CSK4 (1µg/ml) for 4 hours. RNA was analysed by Agilent cDNA microarray gene expression profiling. Heatmap representation of the mean relative expression values of most cytokines/chemokines **(A)** or of cytokines/chemokines analysed by luminex analysis (see Figure 5-9) is shown (mean of 3-13 donors).

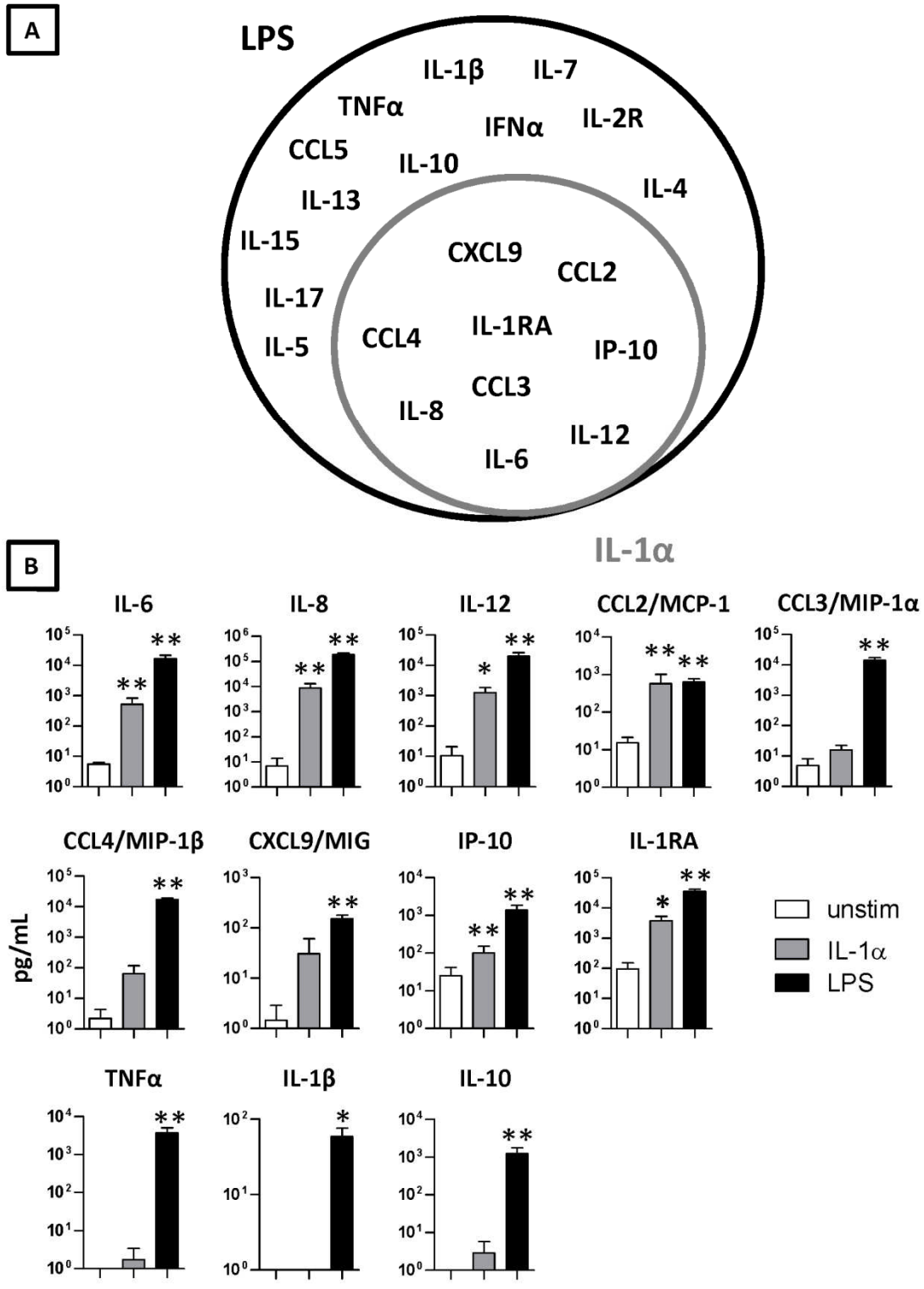


Figure 5-9: IL-1α activation of DCs leads to proinflammatory cytokine release.

DCs were stimulated with 10ng/ml IL-1α or 100ng/ml LPS (*E. coli*) for 20 hours. Supernatants were collected and analysed for 25 different cytokines/chemokines using a Luminex platform. **A:** Venn-diagram. **B:** Representative data. (n=4), data are shown as mean+SEM; n.s. not significant, * $p<0.05$, ** $p<0.01$ (paired student's t-test).

5.3.5 Identification of gene expression signatures that define the IL-1α response

One way to identify patterns and visualise a multivariate dataset is to perform a principal component analysis (PCA). This mathematical procedure reduces the number of variables into principal components (PC) or dimensions in order to highlight similarities or differences within the dataset. The greatest variance is by definition found in PC1, with subsequent components showing successively smaller variances. The PC score is derived from a weighted linear sum of gene expressions (with relative weights termed the loading of each gene). The mean principal component scores for unstimulated DC and the different treatment groups are shown for PC one to four (Figure 5-10A) and the relative expression of the genes with the top 20 loadings are shown in Figure 5-10B. The scores in the first dimension are reflective of the relative magnitude of the response, with LPS furthest away from unstimulated cells, and IL-1α and Pam_3CSK4 stimulated samples intermediate between unstimulated and LPS (Figure 5-10A).

This indicates that the expression of a subset of genes leads to similar qualitative changes after Pam_3CSK4, IL-1α and LPS stimulation, albeit the quantitative response is larger in response to LPS. The relative expression levels in Figure 5-10B confirm these findings and point towards proinflammatory cytokines and chemokines as contributing strongly to PC1. Functional annotation of the 1000 genes with the highest weightings in this dimension similarly indicates a strong bias towards inflammation/cytokine activity (Figure 5-11).

In contrast to PC1, PC2 shows poor discrimination between experimental groups, and is dominated by differences between different unstimulated samples (note large error bars in this dimension), which may arise either from genuine heterogeneity

between individuals, or from experimental variation in sample preparation. The most significant functional cluster in this dimension was cell death associated genes (Figure 5-11), suggesting that viability of the cells at the time of RNA extraction may have a played a role in causing this contribution to variance.

Interestingly, both PC3 and PC4 define dimensions in which IL-1α diverges from LPS, Pam_3CSK4 and the unstimulated samples (Figure 5-10A). The PC3 score seems to be driven predominantly by a subset of genes selectively downregulated in response to IL-1α (Figure 5-10B). Functional annotation identified enrichment in metallothionein genes and genes involved in chemical homeostasis, an observation discussed further below.

The 20 genes with the highest weighting for PC4 include both up and down regulated genes, and functional annotation showed a strong enrichment for genes whose products are found in the extracellular space, and genes associated with inflammation. (Figure 5-10B).

In summary, IL-1α induces an activation programme in DCs which shows both similarities, and differences to those of two TLR ligands, LPS and Pam_3CSK. In Figure 5-1 to Figure 5-9 we show that IL-1α activates a "classical" DC phenotypic and cytokine maturation response, but that this is quantitatively less strong than the response to LPS. In part this reflects the fact that only a subset of DCs appears to respond to IL-1α, even at saturating concentrations of cytokine. In part, this may reflect a failure to activate the type I interferon amplification loop which is strongly induced by LPS signalling.

However, IL-1α also induces a transcriptional response distinct from TLR receptor signalling, and distinct from classical DC maturation. At a systems level, this IL-1α response is partly captured by the third and fourth components of a PCA analysis. The nature of the IL-1α specific response is discussed further below.

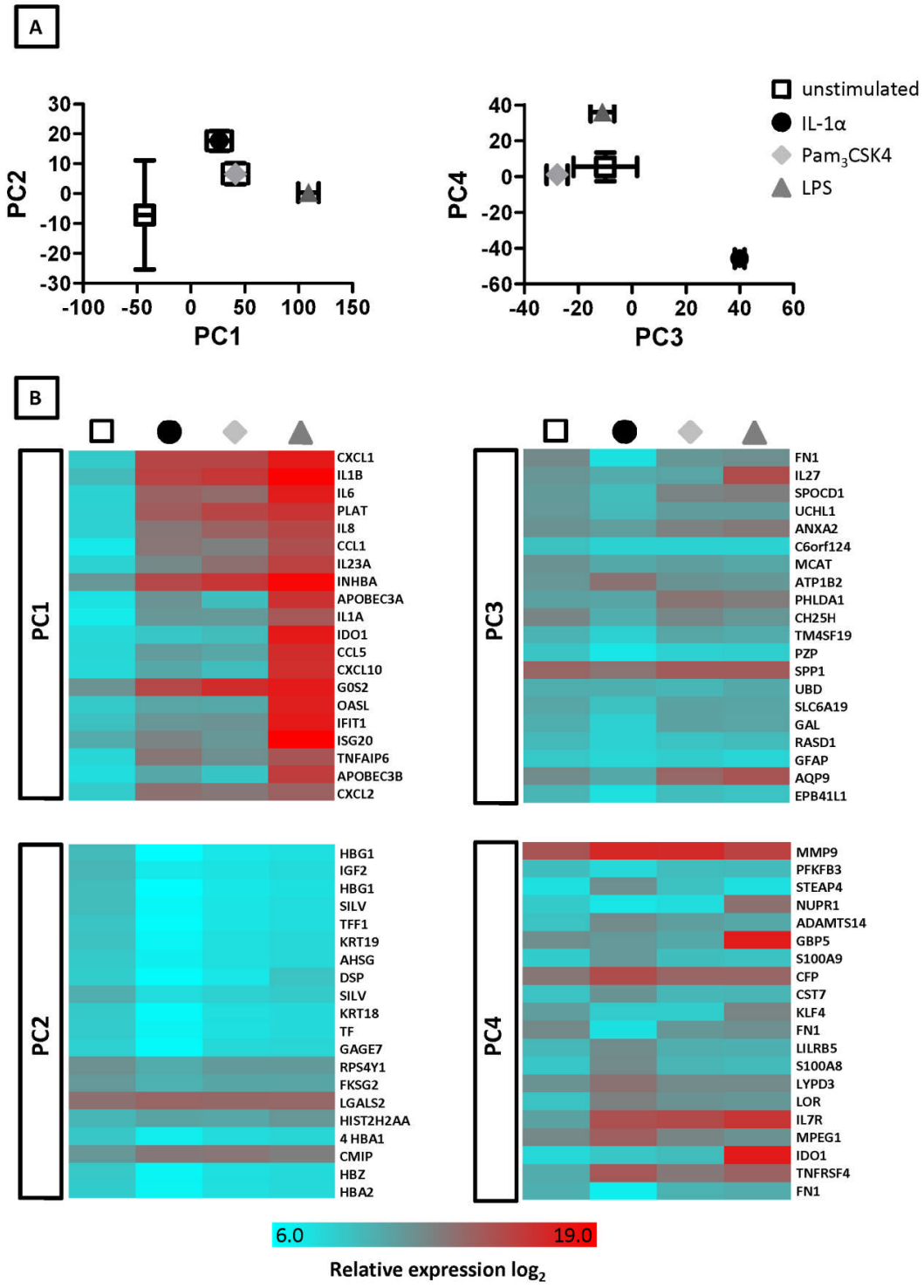


Figure 5-10: Principal component analysis (PCA) of differentially stimulated dendritic cells.

Transcriptional profiling of DCs stimulated for 4 hours with IL-1α (10ng/ml), Pam_3CSK4 (1µg/ml) or LPS (100ng/ml *S. enterica*) was analysed by PCA. **A:** Data points represent mean ± SEM of PC scores from the different donors in each group. **B:** Heatmap of relative gene expression levels for the top 20 genes which account for the greatest variance in principle component (PC) one to four are shown. Data are derived from the mean expression values of three to thirteen separate donors using cDNA microarray gene expression profiling.

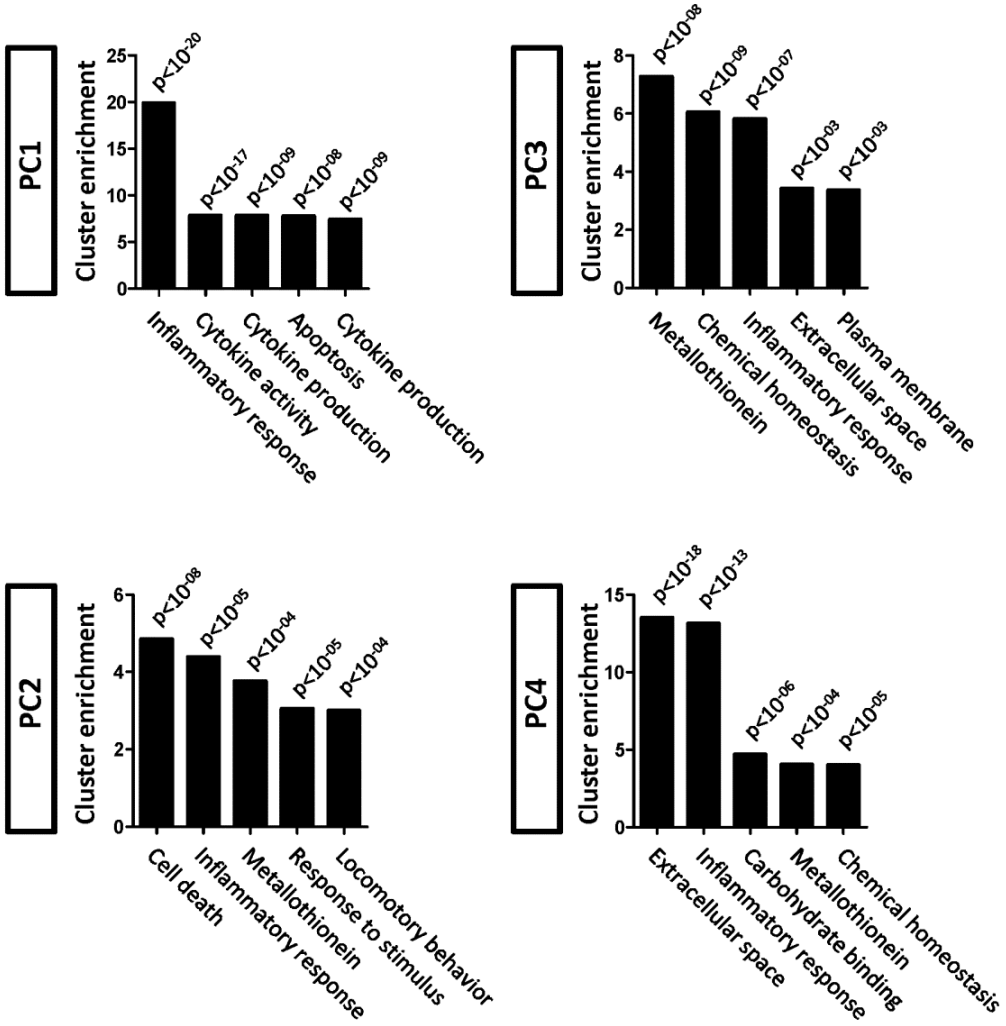


Figure 5-11: Significantly enriched gene ontology groups within PC 1-4.

Transcriptional profiling of DCs stimulated for 4 hours with IL-1α (10ng/ml), Pam_3CSK4 (1µg/ml) or LPS (100ng/ml *S. enterica*) was analysed by PCA. Functional annotation clustering of the top 1000 genes accounting for the greatest variance in the first four PCs was used to indentify enriched gene ontology groups using the online tool DAVID (Modified Fisher's Exact Test).

5.3.6 Further analysis of the IL-1α specific transcriptional response

In addition to functional cluster annotation, we analysed the thousand genes with highest weightings in each component for enriched transcription factor binding sites. This analysis was carried out separately for genes up- or downregulated by IL-1α. The enrichment for the PC3 and PC4 associated genes was much weaker than those for the first two components. The most notable feature was enrichment in zinc-finger TF associated with the downregulation of genes especially in PC2 and PC4 (Figure 5-12).

As shown above (Figure 5-11), PC2 showed functional enrichment for metallothionein. The expression of individual metallothioneins and some of the other genes that were associated with this functional cluster in DAVID are shown in Figure 5-13. IL-1α induced downregulation of many metallothioneins, in contrast to LPS which strongly upregulated many members of this family (Figure 5-13). MT1X and MT2A were the only exceptions as their expression was slightly increased after IL-1α treatment, albeit not as strongly as after LPS stimulation. Most of the other genes that clustered with the metallothioneins were also downregulated after IL-1α stimulation.

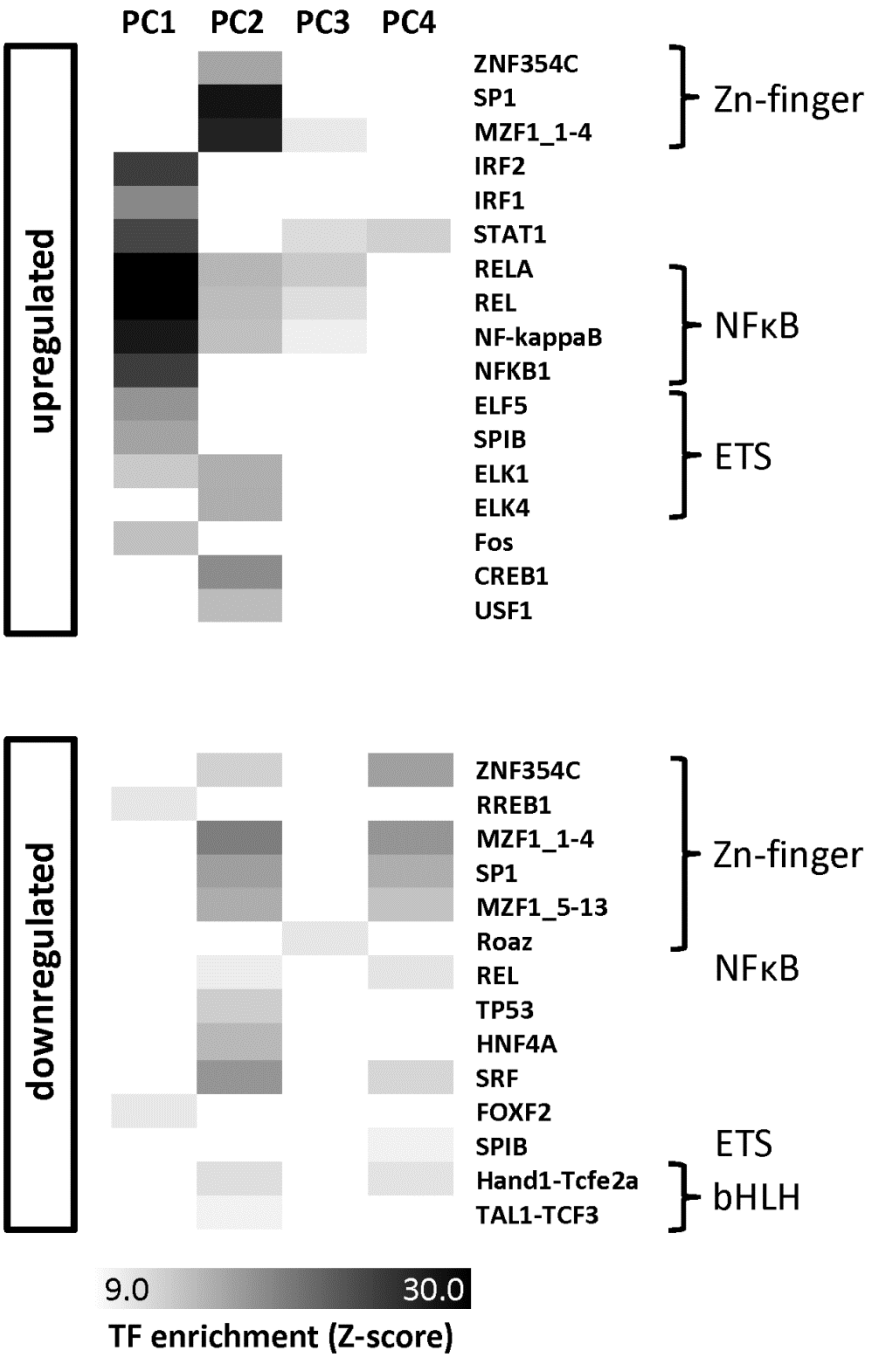


Figure 5-12: Overrepresented transcription factor binding sites in PC 1-4.

Transcriptional profiling of DCs stimulated for 4 hours with IL-1α (10ng/ml), Pam_3CSK4 (1µg/ml) or LPS (100ng/ml *S. enterica*) was analysed by PCA. Transcription factor (TF) binding site enrichment analysis was performed on the top 1000 genes accounting for the greatest variance in the first four PCs using the online bioinformatics tool oPOSSUM. TF binding sites with a Z score higher than 10 are shown.

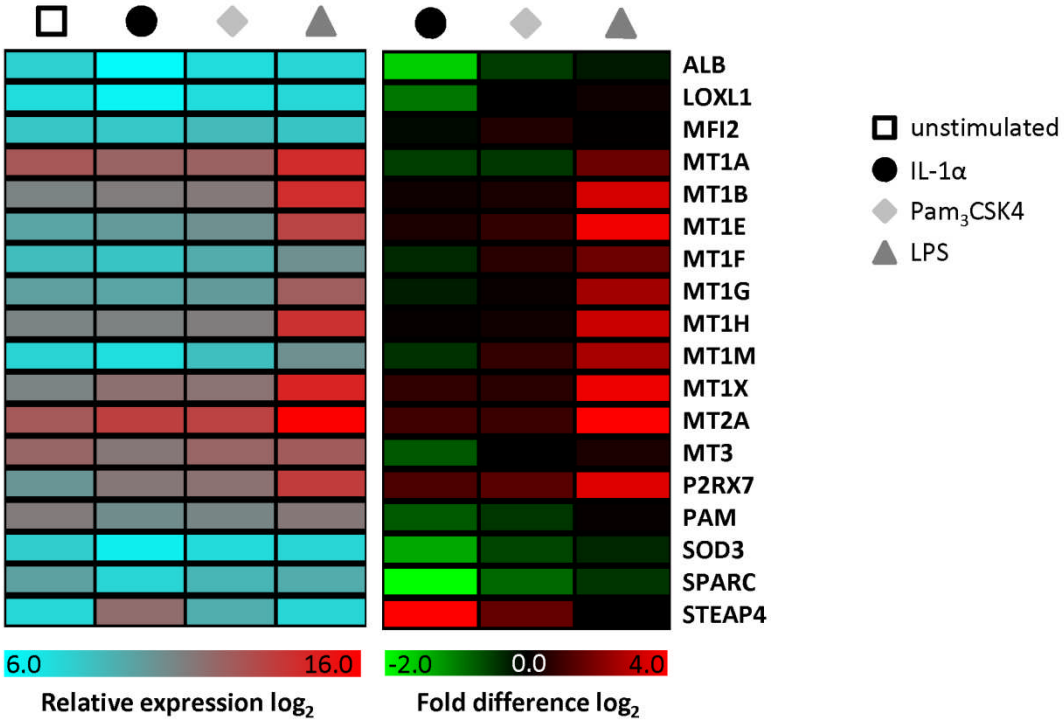


Figure 5-13: Metallothionein expression is not upregulated following IL-1α stimulation.

Transcriptional profiling of DCs stimulated for 4 hours with IL-1α (10ng/ml), Pam_3CSK4 (1µg/ml) or LPS (100ng/ml *S. enterica*) was analysed by PCA. Functional annotation clustering of the top 1000 genes accounting for the greatest variance in the first four PCs was used to indentify enriched gene ontology groups (Figure 5-11). Genes associated with metallothioneins were found amongst the top five clusters in PC2, PC3 and PC4. Expression matrix showing the mean relative gene expression values as well as the mean fold change compared to unstimulated DC for genes that clustered together in "metallothionein" groups in PC2-PC4.

In order to further investigate the IL-1α specific transcriptional response we analysed the 545 genes (Figure 5-4) that changed significantly only after IL-1α treatment by functional annotation clustering (Figure 5-14). The second highest cluster was associated with inflammation and the genes that accounted for the enriched cluster are listed in Figure 5-15. Inflammatory genes that were downregulated after IL-1α stimulation included fibronectin, complement component 3 and carrier molecules, such as albumin, transferrin and apolipoprotein A-II. Upregulated were the chemokines CXCL3 and CXCL14, which both attract monocytes and the cytokine IL-32. Furthermore, the expression of the proinflammatory proteins S100A8, S100A9 and S100A12 was augmented as well as the expression of several complement components

and metallopeptidases (Figure 5-15). As several metallopeptidases seemed to be upregulated by IL-1α (as a general observation while analysing the microarray data) the expression of the matrix metalloproteinases (MMPs) was studied more closely (Figure 5-16). The repertoire of the metalloproteinase family transcriptional response was remarkably stimulus specific. LPS induced an increased expression of MMP7 and MMP19, whereas Pam_3CSK4 stimulation increased the expression of MMP7, MMP9, MMP12 and MMP19. IL-1α stimulation did not result in augmented MMP7 expression, however MMP 9, 10, 12, 14, 19 and 25 expressions were strongly upregulated.

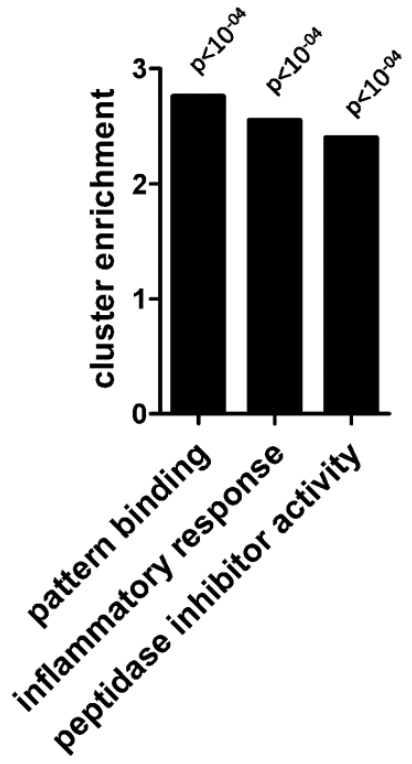


Figure 5-14: Genes that changed uniquely following IL-1α stimulation.

Transcriptional profiling of DCs stimulated for 4 hours with IL-1α (10ng/ml), Pam_3CSK4 (1µg/ml) or LPS (100ng/ml *S. enterica*). Data for the stimulated DCs are derived from three or four different experiments using different donors and were compared to unstimulated DCs from 13 different experiments using different donors. Significant changes were defined as changes in expression compared to unstimulated cells (t-test, $p<0.05$) and >2-fold difference. Genes that changed significantly following IL-1α stimulation, but not after LPS and Pam_3CSK4 treatment were analysed using the functional annotation clustering online resource DAVID (Modified Fisher's Exact Test).

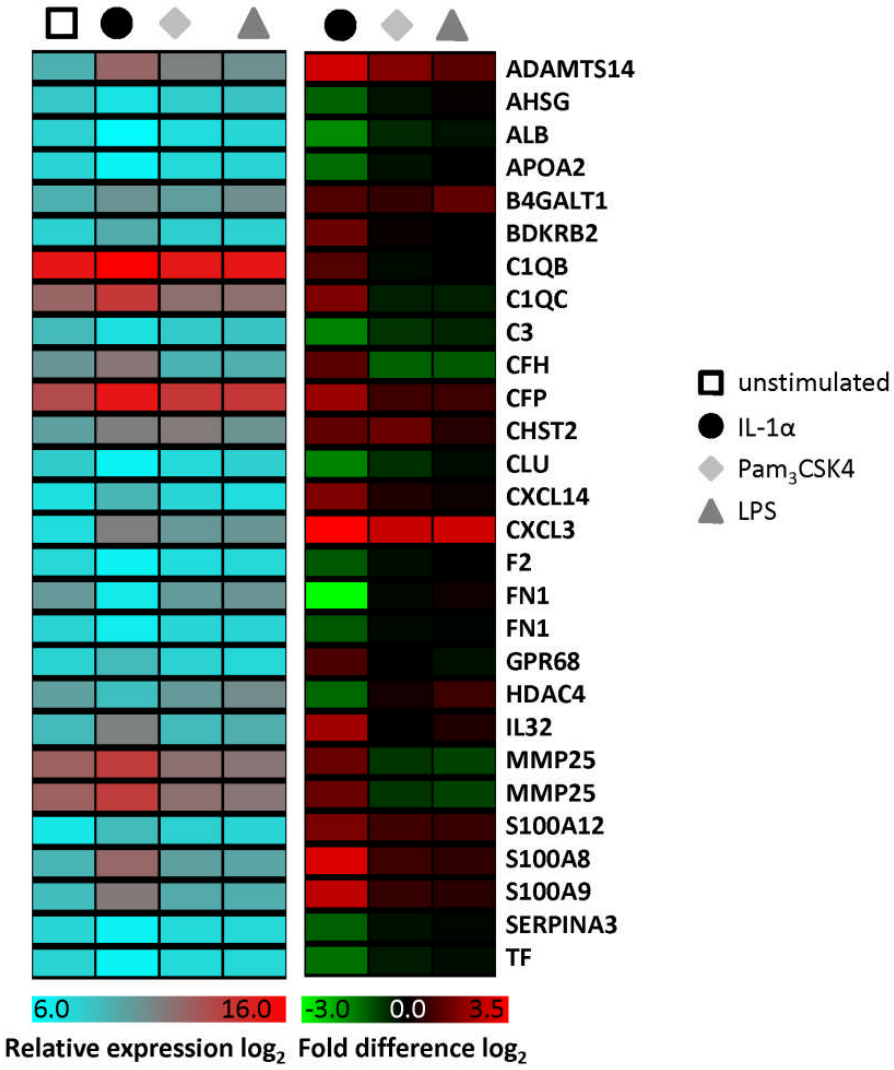


Figure 5-15: IL-1α augments and attenuates the expression of inflammation markers.

Transcriptional profiling of DCs stimulated for 4 hours with IL-1α (10ng/ml), Pam_3CSK4 (1µg/ml) or LPS (100ng/ml *S. enterica*). Genes, which changed significantly ($p<0.05$, >2-fold) following IL-1α stimulation and were unique compared to LPS and Pam_3CSK4 stimulation were analysed by functional gene ontology clustering (Figure 5-14). An expression matrix showing the mean relative gene expression values as well as the mean fold change compared to unstimulated DCs of genes found in the inflammatory response cluster (Figure 5-14) is shown.

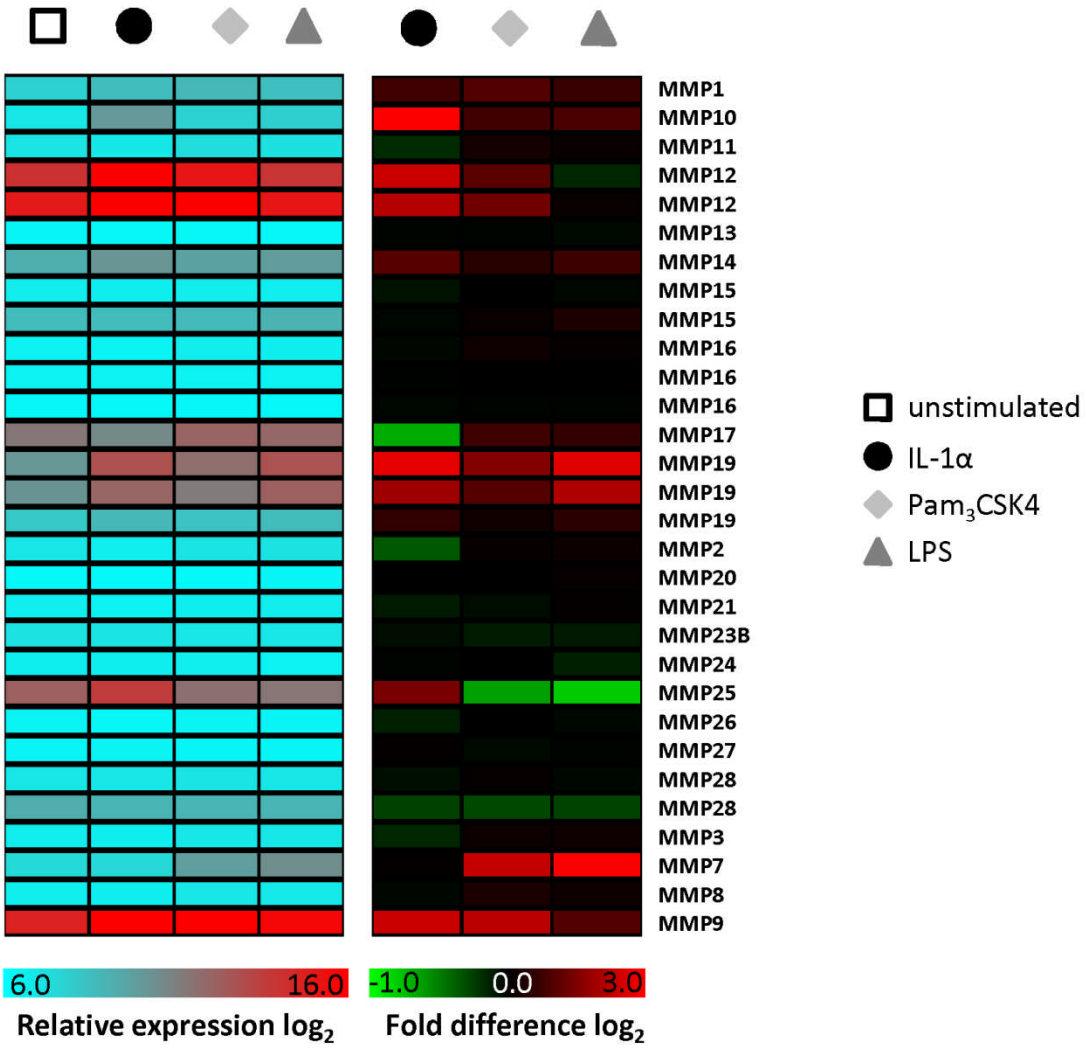


Figure 5-16: IL-1α stimulation increases the expression of MMP.

Transcriptional profiling of DCs stimulated for 4 hours with IL-1α (10ng/ml), Pam_3CSK4 (1µg/ml) or LPS (100ng/ml *S. enterica*). Expression matrix showing the mean relative gene expression values as well as the mean fold change compared to unstimulated DC for all matrix metalloproteinases (MMP).

5.4 Discussion

IL-1α seems to play a crucial role especially in the skin, where it is stored in large quantities in KCs ready to be released upon cell damage. It has been reported previously that KC-derived IL-1α can alarm neighbouring cells, such as surrounding KCs and possibly fibroblasts in the dermis, and induce a proinflammatory response (Bashir et al., 2009; Gahring et al., 1984; Larsen et al., 1989). However, the capacity of IL-1α to stimulate DCs has hardly been investigated, and of the few studies available most were done using mouse DCs. Furthermore, most of these studies can be dated back to the 80s, when the available technology was more restricted than today. Our data from the previous chapter suggest that IL-1α released by chemical-treated KCs contributes to DC activation, which might be a crucial mechanism to prevent infection when the skin barrier is breached.

First we investigated whether IL-1α stimulation of DCs increased typical surface maturation markers. As expected, we found CD80, CD83, CD86, MHC class II, PD-L1, PD-L2 and CD54 (ICAM-1) to be upregulated (Forster et al., 1999; Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996). However, compared to LPS either the MFI was lower, meaning that fewer molecules were expressed per cell or fewer cells expressed the markers. The expression of CD83 and CD86 was studied more closely. Increasing concentrations of LPS lead to an increasing number of DCs expressing CD83 and high levels of CD86 (similar to IL-1α). At saturating LPS concentrations all cells express CD83 and show a high MFI for CD86. Interestingly, saturating concentrations of IL-1α only resulted in half of the cell population being $CD83^{+}$ and $CD86^{hi}$. Therefore, IL-1α itself cannot be the limiting factor. The cells that do express CD83 are also $CD86^{hi}$, suggesting that somehow only half the DC population seems to respond to IL-1α. Wondering if differences in the expression of the IL-1RI might account for

divergent response, we stained DCs before and after IL-1α stimulation with CD83 or CD86 and IL-1RI. The expression of IL-1RI was significantly attenuated after IL-1α stimulation but there was no correlation between CD83/CD86 expression and expression of IL-1RI. In order to transduce a signal IL-1α needs to bind to the IL-1RI as well as the IL-1RAcP. Furthermore, the effects of IL-1α are tightly controlled by the production of IL-1RA and the decoy receptor IL-1RII. Unstimulated DCs constitutively release about 100 pg/ml IL-1RA. It is not clear however, how this could have a selective effect on only half the population. The expression and/or release of IL1-RII and IL-1RAcP remain to be studied and might provide more insight into this phenomenon.

IL-1α and LPS stimulated DCs had the ability to induce allogeneic T cell proliferation. Furthermore, both induced IFN-γ and IL-13 production in allogeneic DC:T cell cocultures. The ratio of IFN-γ to IL-13 production was higher following IL-1α stimulation, which might have been expected as IL-1α stimulated DCs released the Th1 polarising cytokine IL-12, but not the Th2 polarising cytokines IL-4, IL-5 and IL-13, which were released after LPS stimulation. IL-17 production was only augmented during coculture with LPS stimulated DC.

5.4.1 DC stimulated with different innate stimuli share core responses

Next, we wanted to compare the transcriptional profiles of DCs after stimulation with IL-1α, LPS or Pam_3CSK4 for 4 hours. The response to LPS was quantitatively about twice as large as after IL-1α or Pam_3CSK4 stimulation, as measured by the number of genes that were significantly up or downregulated. This was also reflected in the PC1 score, which discriminated between stimuli and reflected the quantitative measure of the response.

The induction of a core maturation process in DCs by IL-1α was confirmed at both RNA message and protein level, by examining a representative set of cell surface molecules and secreted cytokines/chemokines. The gene expression and protein levels corresponded very well, despite the fact that LPS from *S. enterica* was used for the transcriptional profiling experiments, while LPS from *E. coli* was used in all other experiments. Most surface markers, cytokines and chemokines showed augmented expression on RNA and protein level after LPS stimulation compared to IL-1α or Pam_3CSK4 (Le Bert et al., 2011). One exception was CCL2 (MCP-1), where the amounts of protein released after IL-1α and LPS stimulation were the same, whereas the gene expression levels were lower in the IL-1α treated samples. The relationship between gene transcription and protein production is not always straight forward, however. The expression of IL-1β and TNF-α after IL-1α or Pam_3CSK4, for example, was high compared to other genes. However, both cytokines were only released following LPS stimulation, but not after IL-1α or Pam_3CSK4 exposure (Le Bert et al., 2011). IL-1β however is produced as pro-IL-1β which needs to be cleaved by Caspase-1 in order to be secreted, and TNF-α needs to be cleaved off the membrane by ADAM17 (tumor necrosis factor-α-converting enzyme-TACE) in order to be released. Expression of these enzymes was more than 2-fold upregulated after LPS treatment, but not after IL-1α or Pam_3CSK4. Post-translational regulation may therefore play a key role in release of these cytokines.

Qualitative differences in the gene expression profiles induced by the different stimuli under study can be divided into a common core response and a stimulus-specific response. This concept was highlighted in previous publications where the transcriptional responses of DCs to different pathogens (bacteria, viruses and fungi) or the corresponding TLR ligands or 77 collected host-pathogen interactions were

compared (Amit et al., 2009; Huang et al., 2001; Jenner and Young, 2005). Signalling via TIR-domain containing receptors will result in the translocation and activation of common transcription factors, like NFκB and therefore lead to activation of a shared core response (Huang et al., 2001). Indeed, we found that a set of 483 genes was commonly regulated following IL-1α, Pam_3CSK4 and LPS stimulation. Functional annotation clustering identified genes involved in metabolic processes and cell homeostasis to be significantly downregulated and genes involved in inflammatory/defense response, cytokine production and regulation of apoptosis to be significantly augmented, in line with larger studies (Amit et al., 2009; Huang et al., 2001; Jenner and Young, 2005). Binding sites for TFs of the NFκB and ETS family were enriched in the significantly upregulated genes following treatment with the three stimuli. PC1 reflected this shared response as the stimulated samples clustered away from the unstimulated samples in the same direction. Many cytokines and chemokines were found to contribute to PC1 and we were able to confirm on expression and protein level that the response to IL-1α and LPS is qualitatively similar for a certain set of genes, albeit more pronounced in response to LPS. This is not surprising as IL-1α and Pam_3CSK4 only engage one of the TIR adaptor molecules, MyD88, which mainly results in the activation of NFκB. TLR4 signalling however, is mediated not only by MyD88 but also via TRIF, leading to activation of IRF3, which activates interferon-responsive genes (Amit et al., 2009) (Figure 1-3). The combination of both signalling cascades might activate positive feed-forward mechanisms ensuring sustained or augmented gene expression of NFκB regulated genes. Indeed, it has been suggested that IRF3 can function as a co-activator for p65 and vice versa (Leung et al., 2004; Ogawa et al., 2005; Wietek et al., 2003).

Together with the c-jun/ATF-2 heterodimer, NFκB and IRF3 assemble at the IFN-β promoter and induce transcription of the IFN-β gene (Randall and Goodbourn, 2008). It has been suggested that the "early IFNs", IFN-β and IFN-α4, induce a positive feedback loop which results in the expression of further TFs, such as IRF1, IRF7 and IRF9 (Honda et al., 2005; Matsuyama et al., 1993; Sato et al., 1998). These TFs allow a sustained expression of the "early IFNs", as well as induction of the "late IFNs" (IFN-α). Indeed, we found augmented expression of IFN-β, as well as IRF1, IRF7 and IRF9, only following LPS stimulation. Binding of type I IFNs to the interferon-α/β receptor (IFNAR) results in heterodimerisation of STAT1 and STAT2, which together with IRF9 initiate the transcription of genes containing Interferon-Stimulated Regulatory Elements (ISRE). Genes that were significantly upregulated following LPS stimulation showed enrichment for TF binding motifs for STAT1, IRF1 and IRF2. These motifs were not enriched in the upregulated genes following Pam_3CSK4 treatment and only slightly after IL-1α stimulation. This suggests that the IFN feedback loop amplifies the transcriptional response following LPS stimulation inducing a more activated/inflammatory DC phenotype than after IL-1α or Pam_3CSK4 stimulation.

5.4.2 Differential gene expression responses induced by IL-1α stimulation

In an attempt to identify gene expression changes that were unique to IL-1α stimulation we analysed PC2-PC4 further, as IL-1α stimulated samples had clustered away from the other samples in these components. GO terms that were associated with these components were (1) chemical homeostasis, metallothioneins and (2) inflammatory responses, extracellular space.

(1) Metallothioneins can bind a variety of metals, but have especially been associated with zinc homeostasis. It has been shown before that LPS induces

metallthionein expression and lowers the available intracellular free zinc (Kitamura et al., 2006; Leibbrandt and Koropatnick, 1994). Available zinc has been shown to diminish DC activation (Kitamura et al., 2006). Lower expression of metallothioneins after IL-1α and Pam_3CSK4 stimulation might thereby result in higher levels of available zinc compared to LPS stimulated cells and lower expression of inflammatory markers. Interestingly, zinc has been reported to inhibit cyclic nucleotide phosphodiesterase activity and thereby suppress TNF-α and IL-1β production (von Bulow et al., 2005). TLR-induced changes in zinc homeostasis might therefore contribute to augmented DC activation following LPS stimulation. Zinc is an essential component for many proteins. Higher levels of available zinc might therefore preferentially promote their function. Zinc-finger TFs, which require zinc for their function, were also enriched especially for the downregulated genes in PC2 and PC4. One could therefore hypothesise that higher levels of available zinc following IL-1α stimulation enable the downregulation of a certain set of genes through the binding of zinc-finger TFs. Additionally, we observed that several zinc-binding MMPs showed augmented expression after IL-1α stimulation.

An interesting connection between zinc regulation and skin inflammation has been reported in acrodermatitis enteropathica patients. Individuals suffering from this disease show a severe zinc-deficiency which is thought to be caused by mutations in the zinc transporter SLC39A4, preventing zinc uptake in the small intestine (Kury et al., 2002). It has been shown that DCs treated with the zinc-chelating reagent TPEN (N,N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine) show augmented surface expression of CD86 and MHC class II (Kitamura et al., 2006). This finding favours the hypothesis that zinc-deficiency leads to DC activation itself, as well as renders DCs more sensitive to microbial infections. Both mechanisms could contribute to an enhanced skin inflammation.

(2) Next, we studied whether genes uniquely regulated by IL-1α were associated with inflammation. We found proinflammatory molecules like S100A8, 9 and 12, IL-32, CXCL3 and CXCL14 being upregulated. This might indicate that monocytes are more attracted by IL-1α stimulated DCs. However, CCL3 and CCL4 release is augmented after LPS stimulation, which might compensate for that. Furthermore, several complement components and the earlier mentioned metalloproteinases (MMP and A disintegrin and metalloproteinase (ADAM)) were found to show an augmented expression. Amongst the downregulated genes were albumin and transferrin, both proteins that bind zinc in the serum, again suggesting a role for zinc homeostasis. In view of the very selective regulation of MMPs by different stimuli, it might be worth investigating the function of these on DC more closely.

In summary, we find that IL-1α stimulation of DCs induces common core responses that are shared with other TLR ligands, but which are often less pronounced than following LPS stimulation. Distinct IL-1α specific gene expression changes might influence the zinc homeostasis within the cell as well as produce DC with unique abilities to modulate adaptive immune responses.

6 GENERAL DISCUSSION

The release of alarm signals which alert the immune cell to a potential breach of the skin protective barrier is crucial to stimulate protective adaptive immunity, and subsequent migration of immune cells to the site of injury. A large body of literature has established the sentinel role of LCs and dDCs in the skin. The response of DCs to moribund KCs and especially KC-derived IL-1α is therefore likely to be important during skin inflammation. Surprisingly, there is rather little published data on the role of IL-1α on human DCs. In this study, we establish a cytotoxic chemical model of KC insult to explore the hypothesis that IL-1α is a critical danger signal mediating the ability of monocyte-derived DCs to respond to dying KCs.

First, we established a new *in vitro* model where the interaction between KCs and DCs can be studied in the presence of chemicals with irritant and/or sensitising properties. All chemicals tested in this study had irritant properties and therefore induced cytotoxicity of the KCs, which was associated with DC maturation. High HaCaT cell numbers were needed for DC activation, suggesting that soluble factors play an important role. Indeed, the supernatant of the chemical-treated KCs was sufficient to augment expression of the DC activation markers CD83 and CD86. However, coculture with chemical exposed KCs resulted in higher levels of CD83/CD86 expression suggesting that the uptake of dead cell particles or cell-cell recognition contributed to DC activation. We did not observe any differences in terms of sensitisers or irritants on the level of DC activation (expression of surface markers and production of cytokines/chemokines), despite using sensitisers with different strengths and a wide range of concentrations. SDS was the only exception, in that cytotoxicity did not correlate with increased DC activation. Later on, we observed that HaCaT cells killed with SDS did not release any IL-1α. It is most likely that the detergent properties of

SDS interfere with danger signals such as IL-1α, and destroy their DC activation potency.

The danger signals IL-1α and HMGB-1 were found to be released by HaCaTs after chemical induced cytotoxicity. HMGB-1 has been reported to activate DCs (Dumitriu et al., 2007; Yang et al., 2007), but remarkably little is known about the DC response to IL-1α. We confirmed that IL-1α is not only released by necrotic HaCaT cells, but also by primary KCs, using the EpiDerm^{TM} model. We were able to show for the first time that IL-1α, released from moribund KCs, can lead to DC activation. Neutralisation of IL-1α with specific antibodies attenuated DC activation following exposure of supernatants from chemically killed KCs. However, IL-1α neutralisation did not inhibit DC activation completely, suggesting that other danger signals are involved in DC activation in this model.

Interestingly, mixed leukocyte reactions showed attenuated T cell responses following DCs that had been cocultured with DNFB-HaCaTs, but not DMSO-HaCaTs. The protein binding properties of the sensitiser DNFB might account for this effect.

Studying the transcriptional response in DCs following IL-1α stimulation, we found that IL-1α stimulated DCs and induced a proinflammatory phenotype, shown by the (1) upregulation of several DC maturation markers, (2) increased gene expression and protein release of proinflammatory cytokines and chemokines and (3) augmented T cell responses (proliferation and cytokine production). Compared to LPS, the transcriptional response was similar, but not as strong. It has been reported before that different pathogens or TLR ligands induce a shared core response (Amit et al., 2009; Huang et al., 2001; Jenner and Young, 2005). Furthermore, we found that certain groups of genes, such as metallothioneins and MMPs, were differentially regulated

following IL-1α stimulation suggesting that zinc homeostasis may play an important role during DC responses to TLR/IL-1R ligands. We also found the proinflammatory molecules S100A8, 9 and 12, IL-32, CXCL13 and CXCL14 to be specifically upregulated following IL-1α stimulation. Further studies of the functional role of these molecules in DC biology would be valuable.

The release of alarm signals which alert the immune cell to a potential breach of the skin protective barrier is crucial to stimulate protective adaptive immunity, and subsequent migration of immune cells to the site of injury. We propose that during cutaneous immune responses DC maturation and subsequent T cell activation can be achieved via at least three complementary routes. (1) It has been shown in numerous studies that skin DCs (LCs and dDCs) can be directly activated by invading infectious agents, such as HSV (Pollara et al., 2004; Reske et al., 2008). (2) Furthermore, it has been well documented that KCs can actively shape immunological responses in the skin. The release of chemokines and consequent influx of other cellular components of immunity and their modulation by KC-derived cytokines is a crucial part of the skin defence (Feliciani et al., 1996; McKenzie and Sauder, 1990; Tokura et al., 2008). (3) However, under pathophysiological conditions where the magnitude of infection or physical, mechanical or chemical insult induces extensive cytotoxicity in the epidermis, the capacity of KCs and LCs to respond to infection might be compromised. A third mechanism, which has been elaborated in this study, may become important. IL-1α, as well as other danger signals that are released by the dying KCs, diffuse away from the site of insult to inform DCs and tissue cells at adjacent sites that the skin barrier is breached and the risk of incoming infection is therefore increased. Activated DCs and surrounding tissue cells will produce cytokines and chemokines to attract further cells of the immune system to clear a potential infection and initiate wound healing.

Activated DCs can migrate to the LNs and induce an adaptive immune response to foreign antigens sampled in the skin. However, the same pathway might also lead to contact hypersensitivity or other allergic or autoimmune reactions under certain circumstances. Understanding these primary events in the initiation of cutaneous immunity will hopefully contribute to the development of new treatments and the prevention of many skin diseases.

Interestingly, several diseases showing chronic inflammation, such as Crohn's disease or systemic lupus erythematosus, also show symptoms in the skin. Dysregulation of NFκB has been associated with aberrant cell death as well as chronic inflammation in the skin and the intestine (Gerlach et al., 2011; Ikeda et al., 2011; Nenci et al., 2007; Tokunaga et al., 2011). This might be an interesting model to study further the role of IL-1α in the skin.

6.1 Future Work

6.1.1 Initiation of a CHS response

We started this project with the hypothesis that DCs can recognise chemical sensitisers as PAMPs and therefore distinguish them from chemical irritants. However, our results do not support this hypothesis as despite careful titration of the chemicals we did not observe any differences in terms of DC activation following supernatant exposure from sensitiser or irritant treated KCs. This led us to the hypothesis that sensitisers as well as irritants can release danger signals (DAMPs) from surrounding tissue, which lead to DC activation. Indeed, we found IL-1α and HMGB-1 to be released from moribund KCs and were able to show that IL-1α derived from dying KCs contributes to DC activation in our model. All sensitisers used in this study exhibit irritant properties, which were responsible for the release of danger signals. Despite the

fact that irritant potential and sensitiser potency seem to correlate, there are very potent sensitisers that do not show irritant properties. It is far more difficult to imagine how such chemicals initiate CHS responses and it will be important to study how such compounds activate the immune system.

We found that DCs cocultured with chemical-treated HaCaT cells showed augmented expression of CD83 and CD86 compared to DCs treated with the HaCaT cell derived supernatants. It will be interesting to investigate how the cell-cell contact or uptake of the chemical-treated cells induces DC activation, as this is very likely to happen *in vivo* as well.

Many studies have been conducted investigating the activation and migration of LCs out of the epidermis following sensitiser treatment. However, the differential roles of IL-1α versus IL-1β have not been fully elaborated yet. Especially, the differences in IL-1 expression between mouse and man suggest that their roles need to be studied in humans in order to understand their functionality. However, different chemicals might trigger different signals; chemical concentrations and different susceptibilities to allergens are further confounders.

Furthermore, it would be interesting to study the role of other danger signals, such as HMGB-1 in the induction of CHS. Unfortunately, a neutralising antibody for HMGB-1 was not available at the time of this study, and we were therefore not able to probe its role in DC activation in our model.

6.1.2 Development of *in vitro* models to replace the local lymph node assay

Many groups aim to use differential responses of DCs following sensitiser and irritant exposure to discriminate between such groups of chemicals. However, our data

suggest that DCs mainly react to danger rather than specifically to sensitisers. This does not mean that such specific responses do not exist; however even if such specific interactions can be defined, a question will remain about whether they will be robust enough to confidently distinguish sensitisers from irritants with a high accuracy. Furthermore, this response would have to be common to all sensitisers.

In our study we focused on the effect of different concentrations of sensitising and irritating chemicals on HaCaT cells and the subsequent activation of DCs. Thereby, the effect of chemical concentrations ranging from non-toxic to 100% cell death were studied. In order to expand this data set it would be worthwhile to study the kinetics of the chemical-induced HaCaT cell death and the associated DC activation. This might give further insight into chemical-induced cell death and possible differences between different chemicals (e.g. sensitiser versus irritant).

T cells have the ability to distinguish antigenic structures much more specifically than the receptors of innate immunity. Therefore it is not surprising that several groups are now attempting to develop T cell based *in vitro* assays to replace the LLNA.

Intriguingly, we found in our model that DCs cocultured with DNFB-exposed HaCaT cells inhibited allogeneic T cell responses, despite showing a mature phenotype. It would be very interesting to investigate how these DCs inhibited T cell responses.

6.1.3 Dendritic cell response to Interleukin-1α

As mentioned previously, the stimulation of human DCs by IL-1α (and in fact by IL-1β) has not been studied intensively. The IL-1RI and TLRs share the TIR domain, and their downstream signalling pathways are thought to be similar. However, transcriptional profiling revealed that there are stimulus-specific responses as well as a shared core response. The stimulus specific responses for IL-1 (and maybe IL-1α and

IL-1β) and other TLR ligands, such as Pam_3CSK4 have only been poorly defined. More studies dissecting the differential signalling which might account for these stimulus-specific patterns are needed to understand better how APCs are being activated.

Intriguingly, we found that despite saturating concentrations of IL-1α only half of the DC population seemed to respond. We ruled out that IL-1RI expression accounts for this phenomenon. It would be interesting to study further why only some of the cells respond and if a second stimulus, such as IFN-γ priming might lead to full activation of the cells. We only studied the early transcriptional response (4 hours after stimulation) of DCs following treatment with IL-1α, LPS and Pam_3CSK4. A time-course investigating the immediate as well as later responses will no doubt give further insights into IL-1R/TLR-ligand induced transcriptional changes.

Gene expression studies of IL-1α stimulated DCs suggested that zinc homeostasis might play a role in DC activation. Zinc is known to regulate immune processes; however the cellular and molecular targets are poorly defined. Furthermore, the role of zinc during cutaneous immune responses is not well understood. Studying the role of zinc homeostasis in DC activation and the contribution of DCs to skin inflammation, especially in patients with acrodermatitis enteropathica, would be a fascinating line of enquiry. Investigation in this area may also provide insights into the mechanisms leading to zinc-deficiency induced skin inflammation.

Furthermore, we found that MMPs as well as proinflammatory mediators S100A8, 9 and 12, IL-32, CXCL3 and CXCL14 were differentially regulated following IL-1α stimulation. It would be interesting to study their roles further, especially during ACD.

Dysregulation of NFκB is associated with excess cell death and chronic inflammation in different epithelia. It would be worth investigating if IL-1α is released by the dying cells and therefore contributes to the inflammatory milieu.

6.1.4 Conclusion

Our study set out to define a sensitiser–specific interaction between DCs and chemically treated KCs and perhaps to use this as a basis for a novel *in vitro* assay for sensitiser activity. However, we failed to find evidence for such a selective pathway. Instead we found a crucial relationship between KC cytotoxicity and DC activation. On investigation, we found that IL-1α release from dying KCs is one key mediator of this interaction. We speculate that IL-1α release by KCs may be a general "salvage" pathway to stimulate DC migration and adaptive immunity when the local barrier function of the skin has been compromised.

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